Emerging Canine Tick-borne Diseases in Australia and Phylogenetic Studies of the Canine Piroplasmida

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This thesis is presented for the degree of Doctor of Philosophy of Murdoch University

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I declare that this thesis is my own account of my research and contains as its main content, work that has not previously been submitted for a degree at any tertiary education institution.

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ABSTRACT

Canine tick-borne diseases are an emerging problem within Australia and throughout the world. This thesis investigates *Babesia gibsoni* and *Anaplasma platys* infections in dogs in Australia and also explores the evolutionary relationships and taxonomy of the canine piroplasm species and the members of the order Piroplasmida.

A nested PCR-RFLP assay was developed for the detection and differentiation of the canine piroplasm species and was found to have a high detection limit, capable of detecting a 2.7 x 10^{-7} % parasitaemia or the equivalent of 1.2 molecules of target DNA. Detection of piroplasm DNA applied to Whatman FTA® classic cards using nested-PCR was found to have a lower detection limit than when using DNA extracted from whole blood but higher than IsoCode® Stix or QIAamp extraction from filter paper based techniques. The nested PCR-RFLP assay was further evaluated for the detection of *B. gibsoni* infection in dogs being exported from Australia to New Zealand and compared to the current screening methods, the Immunofluorescent Antibody Test (IFAT) and microscopy. Of 235 dogs screened, 11 were IFAT positive, 1 was microscopy positive and 3 were PCR positive for *B. gibsoni*, highlighting the discordance that exists between various detection techniques. Replacing microscopic examination of blood smears with PCR-RFLP is suggested for screening dogs entering New Zealand, in addition to revising the current IFAT cut-off titre to minimize false positive results. The first case of *B. gibsoni* in New South Wales is also reported.

A study was also conducted to further investigate the recent discovery of *B. gibsoni* in Australia and the association of this infection with American Pit Bull Terriers in an epidemiological study. Both American Pit Bull Terriers (n = 100) and other dog breeds (n =

51) were screened for *B. gibsoni* using IFAT and PCR-RFLP. A questionnaire was also completed by each dog owner regarding thethe husbandry and habits these dogs. Fourteen dogs were positive for *B. gibsoni* using IFAT and/or PCR-RFLP and all were American Pit Bull Terriers. Dogs that were male and/or were bitten by or were biters of other American Pit Bull Terriers were statistically more likely to be *B. gibsoni* positive, thus suggesting that blood-to-blood transmission may contribute to the spread of this disease.

Experimental *B. gibsoni* infections were established *in vivo* to investigate the efficacy of combined atovaquone and azithromycin therapy and to determine the detection limits of PCR, IFAT and microscopy during various stages of infection. While atovaquone and azithromycin produced a reduction in circulating parasite levels, it did not cause total eradication, and possible drug resistance also developed in one dog. PCR was found to be most useful in detecting early and acute stage infections, while IFAT was most useful during chronic and acute infections. Microscopy is suggested to be only useful for detecting acute stage infections. This study also describes the detection of *B. gibsoni* in tissue samples during chronic infection for the first time, suggesting possible sequestration of this parasite.

Anaplasma platys has also only recently been reported in Australia and the distribution, molecular-charcterisation, pathogenesis, co-infection with *Babesia canis vogeli* and treatment of infection with doxycycline were investigated. For the first time, *A. platys* is reported in Western Australia, Queensland and Victoria, with each isolate found to be genetically identical on the basis of the 16S rRNA gene. No correlation could be established between *A. platys* infection and the development of clinical signs or pathogenesis and definitive treatment using doxycycline could not be determined.

Isolates of canine piroplasms from various geographical locations worldwide (n = 46), including Australia were characterised on the basis of multiple gene loci to explore the distribution, genetic variation and possible phylogeographical relationships of these species.

Separate genotypes of *B. canis vogeli, B. canis canis* and *B. gibsoni* are suggested and may be correlated to different geographical origins. Characterization of *B. canis vogeli, B. canis canis* and *B. canis rossi* on the basis of the HSP 70 gene and *B. gibsoni* on the basis of the ITS 1, 5.8S rRNA gene and ITS 2 is described for the first time. Elevation of each of the *B. canis* subspecies, with the exclusion of *B. canis presentii*, to separate species is also proposed.

The current paraphyly and taxonomic confusion associated with the members of the order Piroplasmida led to a review of the phylogenetic and taxonomic status of this group of organisms. Phylogenetic relationships are determined using 18S rRNA gene, 5.8S rRNA gene, HSP 70 gene and combined loci analyses. Rearrangement of the Piroplasmida into three families, including the new family Piroplasmiidae is proposed, in addition to the establishment of two new genera, the *Piroplasma* (Patton, 1895) and the *Achromaticus* (Dionisi, 1899). Other proposed schemes of classification and the limitations of phenotypic characteristics in taxonomic classification within the Piroplasmida are also discussed.

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This thesis is dedicated to Alice Mary Paisley-Kerr for cultivating my thirst for knowledge - you are an inspiration.

ABBREVIATIONS AND UNITS

Abbreviations

ANOVA	univariate analysis of variance
AQIS	Australian Quarantine and Inspection Service
BSA	bovine serum albumin
CICT	canine infectious cyclic thrombocytopenia
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
FTA	Flinders Technology Associates
HCT	haematocrit
HGB	haemoglobin
HSP	heat shock protein
ICZN	International Code of Zoological Nomenclature
IFAT	immunofluorescent antibody test
ITS	internal transcribed spacer
LAMP	loop-mediated isothermal amplification method
MAFNZ	Ministry of Agriculture and Forestry, New Zealand
MCV	mean cell volume
MPV	mean platelet volume
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCV	packed cell volume
PLT	platelet number
Q-PCR	quantitative polymerase chain reaction
RCC	red cell count
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
sp.	species (singular)
spp.	species (plural)
TP	total protein
UV	ultraviolet light
WBC	white blood cell count

List of Units

nt	nucleotide
bp	base pair
C	degrees celsius
cm^2	square centimetres
x *g*	times gravity
rpm	revolutions per minute
OD	optical density
nmol	nanomole
pmol	picomole
mol	mole
V	volts
g	gram
mg	milligram
hr	hour
min	minutes
sec	seconds
L	litre
ml	millilitre
□l	microlitre
M	molar
mM	millimolar
mg/ml	milligrams per millilitre
U/ul	Units per microlitre
U	Units

PUBLICATIONS AND CONFERENCES

Publications

The following publications have been drafted for submission:

Jefferies R., Ryan U.M. and Irwin P.J. Development of a nested PCR-RFLP for the detection and differentiation of the canine piroplasm species and its use with filter paper-based technologies

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Jefferies R, Ryan UM, Jacobson L, Baneth G, Mathe A, and Irwin PJ. Proposed re-classification of the *Babesia canis* subspecies, including elevation of each to a species level of classification.

Jefferies R., Ryan U.M. and Irwin P.J. A review of the taxonomic status of the order Piroplasmida

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CHAPTER ONE

Introduction and General Aims

Ticks are capable of transmitting a wide range of pathogens including viruses, bacteria and protozoa, highlighting their importance as vectors of disease for mammals, birds and reptiles. While tick-borne diseases are considered to be 'emerging', the validity of this term has been questioned, as it is not clear as to whether the increased prevalence and distribution of these pathogens is simply a reflection of the improved levels of detection, surveillance and awareness (Telford and Goethert 2004). Changes in climatic conditions and the increase in international travel of both humans and animals are also considered important factors involved in the epidemiology of tick-transmitted diseases (Shaw *et al*. 2001). It is likely that a combination of factors have contributed to both the increased detection, prevalence and distribution of these diseases and the impact of tick-borne diseases on humans, companion animals, livestock and wildlife should not be underestimated (Jongejan and Uilenberg 2004). Tick-borne pathogens are therefore of global significance, further highlighting the need for increased research in a number of key fields including epidemiology, diagnosis and chemotherapy. This thesis investigates emergent tick-borne diseases, with particular emphasis on molecular epidemiology of these infections in domestic dog populations within Australia and also explores the areas of phylogenetics and molecular taxonomy.

1.1 Canine tick-borne diseases

Tick-borne diseases of dogs are a common feature in tropical and subtropical regions (Irwin and Jefferies 2004), however many are also associated with temperate climates (Shaw *et al*., 2001). The main groups of canine tick-borne infections include the protozoan diseases (caused by *Babesia* spp, *Theileria* spp., *Hepatozoon* spp) the rickettsial and bacterial diseases (*Ehrlichia* spp., *Anaplasma* spp., *Rickettsia* spp., *Bartonella* spp., *Coxiella* spp., and *Borrelia* spp.) and the viral infections (tick-borne encephalitis). Co-infections of *Babesia* and *Anaplasma,* along with *Ehrlichia, Bartonella, Hepatozoon*, *Leishmania* and *Rickettsia* species have also been reported in dogs (Rajamanickam *et al*., 1985; Kordick *et al*., 1999; Suksawat *et al*., 2001b; O'Dwyer *et al*., 2001) and may complicate the clinical signs and pathogenesis of infection (Harvey, 1990; Shaw *et al*., 2001). Of the tick borne protozoan pathogens, this thesis investigates the canine Piroplasmida, including both *Babesia* and *Theileria* spp. and the rickettsial pathogen, *Anaplasma platys*. A review of the literature on the canine Piroplasmida is presented in Chapter two and a review of literature on *A. platys* is presented in Chapter three.

Historically, the only tick-transmitted pathogen of dogs reported in Australia was *Babesia canis vogeli* (Hill and Bolton, 1966; Irwin and Hutchinson, 1991), distributed predominantly throughout the northern, subtropical regions. With the recent discovery of *A. platys* (Brown *et al*., 2001) and *Babesia gibsoni* (Muhlnickel *et al*., 2002) canine tick-transmitted diseases are now considered emergent within Australia and this also raises concerns about effective quarantine screening of dogs and biosecurity. Limited study had been conducted on the epidemiology, pathogenesis, prevalence, distribution and control of both pathogens within Australia. This thesis further investigates both *B. gibsoni* and *A. platys* infections in Australia using molecular based detection techniques (Chapters five to nine).

1.2 Molecular phylogeny and taxonomy of the Piroplasmida

In addition to its role in diagnosis, molecular-based characterisation of pathogens, such as the canine piroplasms, has allowed for greater insight into the phylogenetic relationships and molecular taxonomy of these organisms. Considerable confusion currently exists in determining the correct taxonomic description for species of canine piroplasm and for all members of the order Piroplasmida at the species, genus and family levels of classification. The molecular phylogeny and taxonomy of the canine piroplasm species, in addition to all members of the order Piroplasmida are investigated in Chapters ten and eleven.

An overview of each subproject investigated and the inter-relationships between each subproject within this thesis is shown in Figure 1.1.

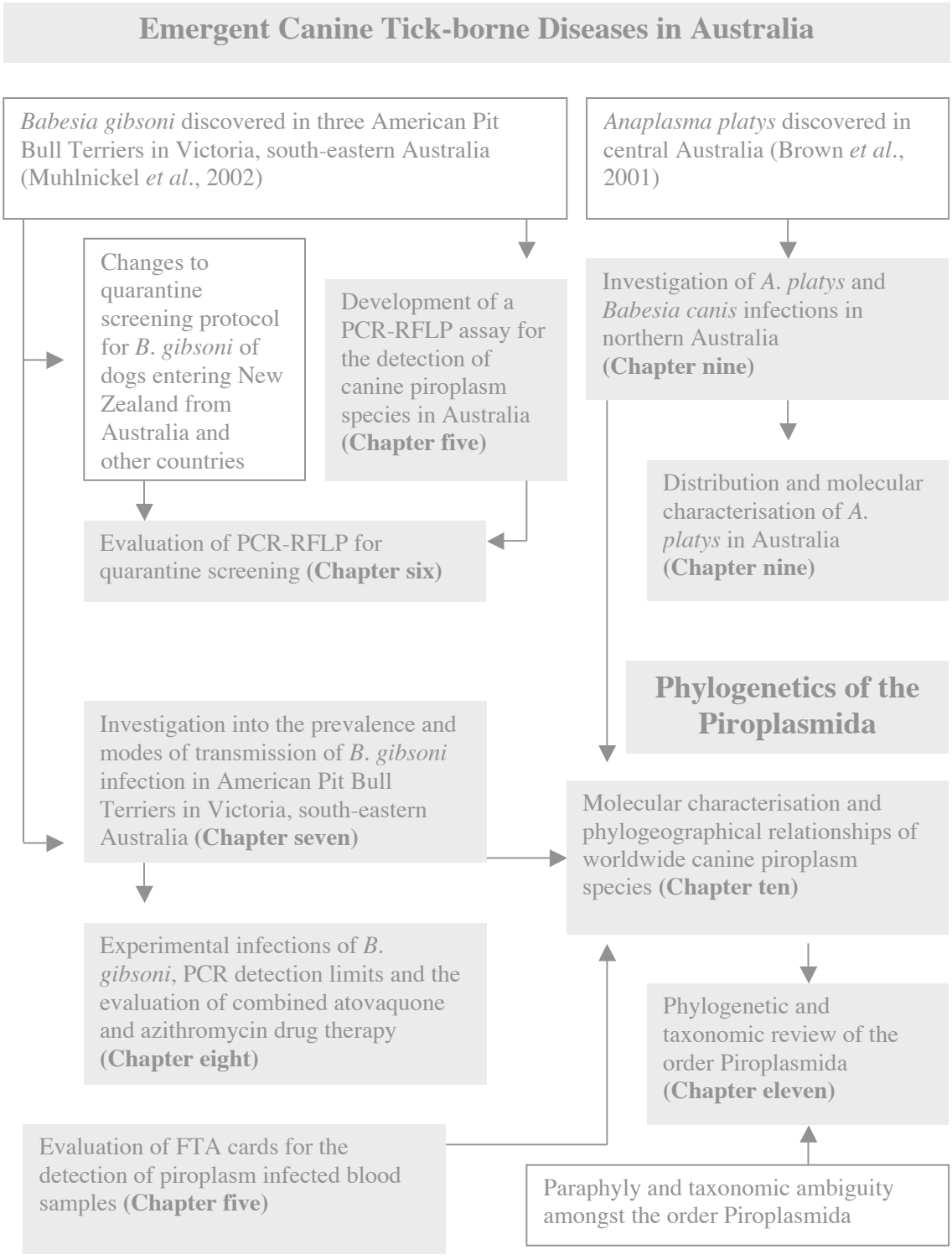


Figure 1.1

Flow diagram representing the relationships between the thesis subprojects

1.3 General aims

1. To develop a PCR-RFLP assay for the detection and differentiation of the canine Piroplasmida species

2. To evaluate Whatman FTA classic cards for the application of canine blood and subsequent use for PCR detection of piroplasm DNA

3. To evaluate PCR-RFLP for quarantine screening of dogs for *B. gibsoni* infection

4. To assess the prevalence and transmission dynamics of *B. gibsoni* infections in American Pit Bull Terriers in Victoria, Australia

5. To investigate the efficacy of atovaquone and azithromycin drug therapy and detection limits of PCR using experimental infections of *B. gibsoni*

6. To determine the distribution of *A. platys* in Australia and molecularly characterise isolates from different geographical locations

7. To investigate co-infections of *A. platys* and *B. canis* and the efficacy of doxycycline treatment

8. To molecularly characterise isolates of *B. canis* and *B. gibsoni* collected worldwide and investigate phylogeographical relationships among isolates

9. To review the phylogenetic and taxonomic relationships of the order Piroplasmida

CHAPTER TWO

Review of Literature on the Canine Piroplasmida

Piroplasmosis is the collective term for diseases caused by 'piroplasms'; intracellular, blood-borne protozoan parasites of the order Piroplasmida. These tick-transmitted diseases, many of which are of veterinary and medical significance, have been described worldwide, in a large diversity of mammals, birds and reptiles. Piroplasmosis is a significant disease of members of the Canidae, with multiple species of piroplasm reported to infect dogs and wild canines. Although some of these piroplasm species cause limited pathogenesis, others can produce severe illness, often leading to death. Identifying the species and subspecies of piroplasm infecting dogs is of importance in the accurate management of the disease including its diagnosis and subsequent treatment.

Piroplasmosis is considered an emerging disease syndrome, with many new species being described and multiple species showing increasing prevalence and worldwide distributions. Whether this increase is due to an increased awareness, the use of more sensitive detection methods, or changing global travel patterns has yet to be determined.

2.1 Taxonomic classification of the canine piroplasms

Members of the order Piroplasmida are apicomplexan protozoa categorized into four main families; Anthemosomatidae, Babesiidae, Theileriidae and Haemohormidiidae (Levine, 1988). The families Babesiidae and Theileriidae are well documented and include the genera, *Babesia, Entopolypoides, Cytauxzoon* and *Theileria*. Historically, multiple genus names have described the Piroplasmida including *Piroplasma*, *Pyrosoma, Apiosoma*,

Nuttallia, Nicollia, Babesiosoma, Smithia and *Rossiella* (Levine, 1988), each of which are no longer generally accepted. It has also been suggested that the genus *Entopolypoides* is synonymous with the genus *Babesia* (Gleason and Wolf, 1974; Bronsdon *et al*., 1999). There is currently no consensus regarding correct species allocation within the order Piroplasmida.

Early classification of these blood-borne piroplasms relied heavily upon examination of their morphological and life cycle characteristics (Allsopp *et al*., 1994). Initial taxonomic classification of the canine piroplasms was on the basis of size and allowed for the separation of two species, the 'large' *Babesia canis* and 'small' *Babesia gibsoni*. Evidently, there are limitations in such a general consignment to a single species on the basis of host specificity and morphological similarity. For example, it has now been noted that some species of *Babesia* are not host specific, such as *B. microti*, which can infect a wide range of vertebrate hosts (Etkind *et al*., 1980; Moore and Kuntz, 1981). Additionally, *B. microti* cannot be reliably differentiated from *B. gibsoni* when examining Giemsa-stained blood smears using light microscopy (Conrad *et al*., 1992).

Molecular characterisation on the basis of conserved gene loci has significantly aided the accurate identification of a species and also allows further discrimination to a genotypic level. The classification of the piroplasms has received renewed attention with the advances in molecular biology and has resulted in the characterisation of more than two canine piroplasm species and the infection of dogs with piroplasm species formerly considered specific to other hosts (Table 2.1).

Piroplasm size	Traditional canine species	Molecular characterisation
Large	*Babesia canis*	*Babesia canis canis* (Carret *et al*., 1999)
(3 – 5 μm)		*Babesia canis vogeli* (Carret *et al*., 1999)
		Babesia canis rossi (Carret *et al*., 1999)
		Babesia sp. (Birkenheuer *et al*., 2004b)
Small	*Babesia gibsoni*	*Babesia gibsoni* (Zahler *et al*., 2000c)
(1 – 2 μm)		*Babesia conradae* (Kjemtrup *et al*., 2000a; Kjemtrup et al., 2005)
		Theileria annae (Zahler *et al*., 2000b)
		Theileria equi (Criado-Fornelio *et al*., 2003a)

Table 2.1

Comparison of traditionally accepted and genetically characterised species of piroplasm isolated from dogs

2.1.1'Large' canine piroplasm species

Babesia canis was first described by Piana and Galli-Valerio (1895) and was historically recognised as the only species of large piroplasm known to infect dogs. Since then, noticeable differences in vector specificity and infection pathology between isolates led to the proposal of three separate subspecies of *Babesia canis* (Uilenberg *et al*., 1989). *Babesia canis rossi* is transmitted by *Haemophysalis leachi* and is reported to have the most severe pathogenic manifestations. *Babesia canis canis*, transmitted by *Dermacentor spp*. can give rise to a moderate clinical disease and *B. canis vogeli* is transmitted by *Rhipicephalus sanguineus*, producing the least severe clinical disease. DNA sequencing has led to confirmation of the proposed three subspecies on the basis of the nuclear small subunit 18S ribosomal RNA (18S rRNA) gene (Carret *et al*., 1999) and Internal Transcribed Spacers (ITS) 1 and 2 (Zahler *et al*., 1998). In addition it has been proposed that the three subspecies are genetically distinct enough to obtain species status (Zahler *et al*., 1998; Carret *et al*., 1999).

A fourth subspecies of *B. canis* has also been proposed, *B. canis presentii,* which was identified in domestic cats from Israel (Baneth *et al*., 2004) and an additional species of 'large' canine *Babesia* has been reported in a Labrador in North Carolina (Birkenheuer *et al*., 2004b). This species remains unnamed.

2.1.2'Small' canine piroplasm species

Patton (1910) was first to describe *Piroplasma gibsoni* as a species, when this small piroplasm was identified within the blood of dogs and jackals in India. This species was later renamed *Babesia gibsoni* and subsequent findings of all small piroplasms in canine blood were also assigned the same species name on the basis of morphology and host specificity (Botros *et al*., 1975). Molecular-based characterisation of various isolates of small canine piroplasms from separate geographical regions of the world has however, identified distinct genetic variants and resulted in the differentiation of the small canine piroplasms into more than one species.

Zahler *et al*. (2000b) first described the existence of a small canine piroplasm from Spain that was genetically distinct from *B. gibsoni* and was most genetically similar to a rodent/human species, *B. microti,* on the basis of the 18S rRNA gene. This isolate was taxonomically classified as a member of the genus *Theileria* and named *T. annae*.

Isolates of small canine *Babesia* from Asia and North America were also compared on the basis of sequence analysis of the 18S rRNA gene (Zahler *et al.,* 2000c). Phylogenetic comparison of these geographically different isolates suggested that the Asian isolates belong to different species to the American isolate (obtained from dogs in California). Further support of the existence of at least three species of small canine *Babesia* was developed when strains from Okinawa, Oklahoma, North Carolina, Indiana, Missouri and Alabama were compared phylogenetically (Kjemtrup *et al*., 2000a). The isolate from

California which has been named *Babesia conradae*[1] (Kjemtrup *et al*., 2005) .was shown to be most closely related to a human species of piroplasm referred to as 'WA1' (Quick *et al*., 1993) (now described as the species *Babesia duncani*). The remaining isolates from Asia and the Midwestern and eastern United States have been classified as *B. gibsoni*, often with the qualifier 'Asian genotype' to avoid further confusion.

Additionally, there have been reports of *Theileria equi*, a species normally only associated with horses, found within dogs (Criado-Fornelio *et al*., 2003a; Criado-Fornelio *et al*., 2004). The pathogenicity of *T. equi* is currently unknown as only four dogs have been reported to be infected, one described as 'symptomatic', while the remaining three were clinically normal.

2.2 Phylogeny and evolutionary relationships among the Piroplasmida

An increased understanding of the phylogenetic relationships among the Piroplasmida has been established through the use of genetic-based analysis. Much confusion still exists over the correct evolutionary relationships among the canine piroplasms and may not be resolved until additional isolates and species are genetically characterised on the basis of multiple gene loci. To date, phylogenetic analyses of the Piroplasmida have concentrated on the conserved 18S rRNA gene.

A limited number of studies have determined the overall phylogenetic relationships between members of the phylum Apicomplexa and is likely to be a reflection of the many thousands of species described (Escalante and Ayala, 1995). Most research has concentrated on selected genera that have medical or veterinary significance. In general, the Piroplasmida have been shown to share a common ancestor with members of genus *Plasmodium*, forming

[1] *Babesia conradae* is synonymous with early reports of *B. gibsoni* described from California (Conrad *et al*., 1991; Conrad *et al*., 1992; Wokniak *et al*., 1997), *B. gibsoni* (Californian genotype) (Zahler *et al*., 2000c; Kocan et al., 2000; Kocan *et al*., 2001, 'Dog from California' (GenBank accession No. AF158702)

a separate clade with the genera *Sarcocystis, Neospora* and *Toxoplasma*. All characterised species of the Piroplasmida (of the families Babesiidae and Theileriidae) form a distinctive, individual clade, separate from all other members of the phylum Apicomplexa. No research has been published on the phylogenetic relationships of the little known Piroplasmida families, the Anthemosomatidae and Haemohormidiidae.

Early phylogenetic classification of the Piroplasmida relied solely on morphological and/or life cycle characteristics. Members of the genus *Theileria* were differentiated from other species of piroplasm by the presence of a tetrad or 'maltese cross' formation of the intraerythrocytic merozoites and the existence of an exoerythrocytic lifecycle stage (Mehlhorn and Schein, 1984). The *Theileria* were also distinguished by transstadial transmission in the tick vector as opposed to the transovarial transmission found to occur in the 'true' *Babesia*, termed the *Babesia sensu stricto* (Mehlhorn and Schein, 1984).

Allsopp *et al*. (1994) first assessed the phylogenetic and evolutionary relationships of the piroplasms on the basis of the 18S rRNA gene of a limited number of species of *Babesia*, *Theileria* and *Cytauxzoon*. This study suggested that most of the *Babesia* spp (the *Babesia sensu stricto)* and the *Theileria* spp. separated into distinct monophyletic clades. A third group containing *Babesia rodhaini, Babesia equi* and *Cytauxzoon felis* was inferred to be ancestral to the only the *Theileria* or both the *Theileria* and the *Babesia sensu stricto* groups. This group was proposed as a new Family, the Nicollidae (Allsopp *et al*., 1994).

Using an increased number of piroplasm species, including newly described human and wildlife piroplasm species from western USA, Kjemtrup *et al*. (2000b) further conducted phylogenetic analysis using the 18S rRNA gene. Four distinct groups were inferred from phylogenetic trees. As in previous studies, the *Babesia sensu stricto* group were distinctly separated from the *Theileria* group. An additional group of piroplasms was reported in this study, termed the western *Babesia* spp. group, which contained wildlife and human

piroplasm spp, in addition to *B. conradae* (Kjemtrup *et al*., 2000b). *Babesia microti* was found to form a fourth clade, ancestral to all other three groups of piroplasms.

The phylogenetic position of the canine piroplasm species in early analyses related solely to *B. canis*. Allsopp *et al*. (1994) found that *B. canis* belonged to the *Babesia sensu stricto* group. Further support for this was provided when *B. canis canis* and *B. canis rossi* were shown to cluster together and that *B. canis vogeli* separated into a monophyletic group with *B. odocoilei* and *B. divergens* (Carret *et al*., 1999). *Babesia conradae* was found to belong to the western *Babesia* spp. group (Kjemtrup *et al*., 2000b).

Further revision of the phylogenetic relationships among the Piroplasmida has been proposed by Criado-Fornelio *et al*. (2003b), with the formation of five distinct groups (Figure 2.1). The most ancestral group of the Piroplasmida has been proposed as the Archaeopiroplasmids, including *T. annae, B. microti, B. rodhaini* and *B. felis*. It is suggested that the remaining *Babesia* and *Theileria* species evolved from the Archaeopiroplasmids to form the Ungulibabesids, Babesids (including *B. canis* and *B. gibsoni*), Prototheilerids (including *B. conradae*) and the Theilerids.

Criado-Fornelio *et al*. (2003b) further speculated that piroplasmids (members of the Piroplasmida) first began to develop as parasites of ticks or mammals about 55-60 million years ago in Africa during the Paleocene, supporting suggestion by Penzhorn *et al*. (2001), that Africa is the likely origin of the piroplasms. It is also suggested that species of Archaeopiroplasmid initially were parasites of rodents then also began infecting ancestors of the carnivores (Criado-Fornelio *et al*., 2003b). A lineage of these piroplasms then developed into the Prototheilerids, infecting primitive carnivores and ungulates. The Prototheilerids, notably a *T. equi* ancestor evolved into two distinct groups, the Theilerids and the Babesids/Ungulibabesids.

Figure 2.1

Distance based phylogenetic tree of the Piroplasmida (adapted from Criado-Fornelio *et al.*, 2003b), Arrows indicate piroplasm species found in dogs (*Babesia* sp – North Carolina, not included)

The phylogenetic position of the 'large' canine piroplasm from North Carolina was suggested to be closely related to the ungulate *Babesia* spp and ancestral to both the *B. canis* subspecies and *B. gibsoni* (Birkenheuer *et al.*, 2004b – Figure 2.2).

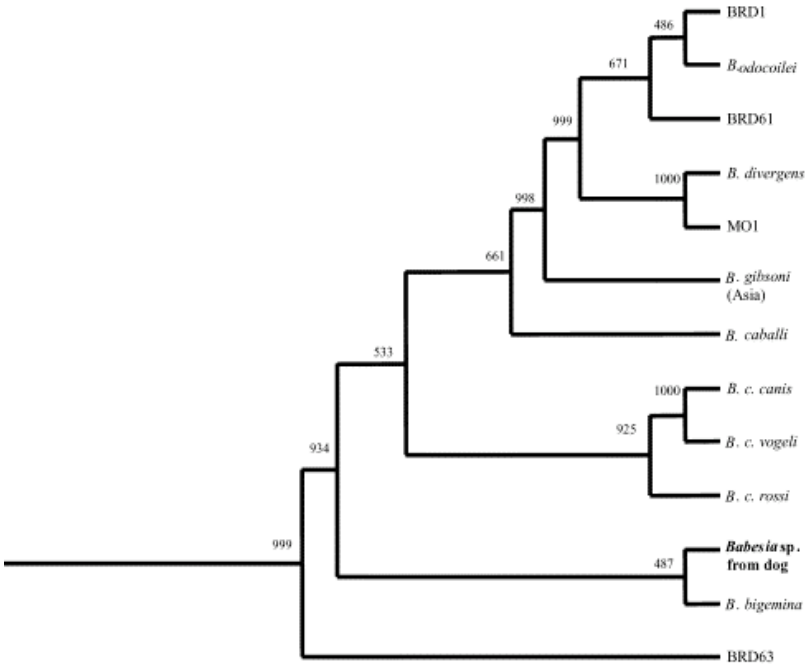


Figure 2.2

Partial phylogenetic tree identifying the evolutionary relationships between the large *Babesia* sp. from a dog (North Carolina) and other *Babesia* species (adapted from Birkenheuer *et al*., 2004b)

2.3 Morphology

2.3.1 'Large' canine piroplasm species

All 'large' species of canine piroplasms are typically 2 – 5 μm in diameter, with differentiation of individual species and subspecies difficult on the sole basis of morphology. Trophozoites of *B. canis* are characterised by a length of approximately 5.0 μm and a width of 2.5 - 3.0 μm and are generally described as large canine *Babesia* (Kuttler, 1988, Conrad *et al.,* 1992). *Babesia canis* piroplasms are generally pear-shaped, occurring singularly or as pairs of dividing trophozoites inside the erythrocyte (Kjemtrup *et al.,* 2000a), but a wide range of morphological characteristics are recognised.

The *Babesia* sp. (North Carolina) described by Birkenheuer *et al*., (2004b), was reported as being polymorphic, typically singular, with occasional two pyriform-shaped organisms

joined at a 90º angle. Parasites ranged in size from approximately 2 μm x 3.5 μm to 5 μm x 6 μm, which again reinforces that differentiation from the other large canine *Babesia* spp. may be difficult, if not impossible on the sole basis of morphology.

2.3.2 'Small' canine piroplasm species

The 'small' piroplasm species, including both *Theileria* and *Babesia*, are typically 0.5 – 3 μm in diameter. The trophozoites of *B. gibsoni* are smaller (1.2 to 2.2 □m), and are therefore referred to as the small canine *Babesia* (Kuttler, 1988, Caspulla *et al.,* 1998, Fukumoto *et al.,* 2000). *Babesia gibsoni* also appear as pleomorphic protozoa, usually round, oval or pear shaped (Conrad *et al.,* 1992, Casapulla *et al.,* 1998). *Babesia gibsoni* is most abundant singly and rarely exists as paired pyriform bodies within erythrocytes (Fukumoto *et al.,* 2000). It has been reported that *B. conradae* and *T. annae* are capable of forming intraerythrocytic tetrads, a feature not witnessed for *B. gibsoni* infection (Kocan *et al.*, 2001).

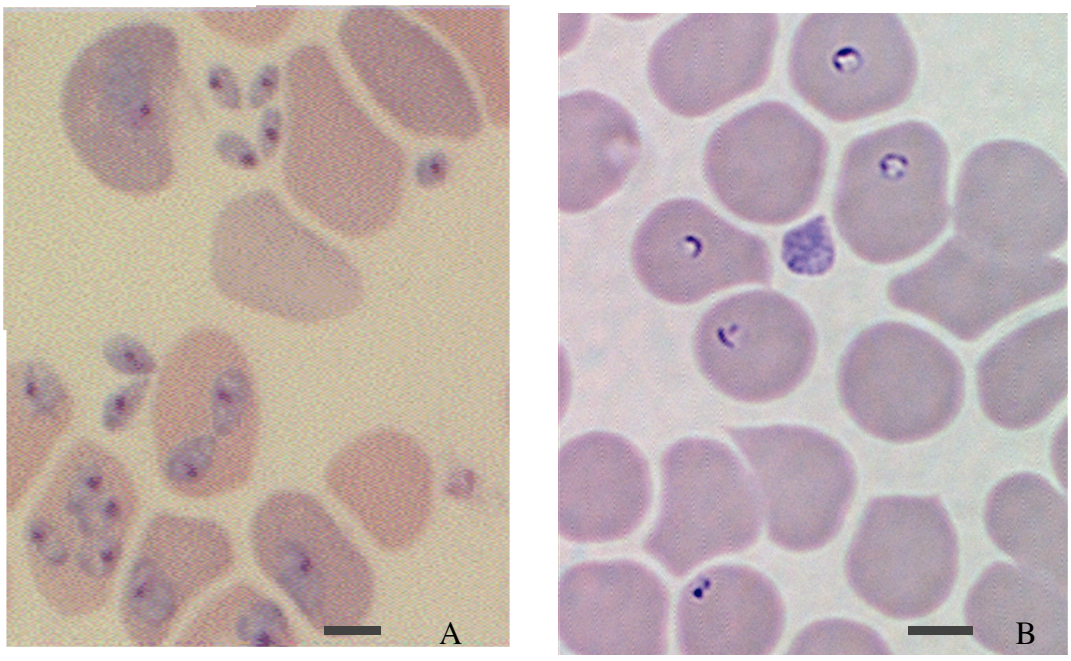


Figure 2.3

Typical morphology of 'large' canine piroplasms (A - *Babesia canis vogeli*) and 'small' canine piroplasms (B – *Babesia gibsoni*) Scale bar represents 5 □m (Images from Jefferies, 2001).

Electron microscopic examination of intraerythrocytic *B. gibsoni*, revealed the presence of four morphologically distinct trophozoite stages; small spheres, small rods, irregular forms lacking pseudo-inclusions and large spheres with pseudo-inclusions (Radi *et al*., 2004).

2.4 Transmission

2.4.1 Tick vectors

Tick species are recognized as being the main vector responsible for the transmission of all species of piroplasm. Each of the species and subspecies of the canine piroplasms is tick vector specific, with each of the *B. canis* subspecies infecting a single and separate tick species. A summary of the known tick species that transmit the different species and subspecies of the canine piroplasms is given in Table 2.2. It is important to recognise that many transmission studies carried out may not be reliable and that a definitive list of tick vector species of the canine Piroplasmida has not been determined.

Piroplasm species	**Tick vector species**	**Citations**
Babesia canis canis	*Dermacentor reticulatus* *Dermacentor marginatus?*	Schein *et al*. (1979); Mehlhorn *et al*. (1980) Jongejan and Uilenberg (2004)
Babesia canis rossi	*Haemophysalis leachi*	Lewis *et al*. (1996)
Babesia canis vogeli	*Rhipicephalus sanguineus*	
Babaesia gibsoni	*Haemophysalis bispinosa?*, *Haemophysalis longicornis* *Rhipicephalus sanguineus?*	Higuchi *et al*. (1991a); (1991b); (1992); (1993a); (1993b); (1995); (1999a); (1999b)
Babesia sp. (North Carolina)	Currently undetermined	
Babesia conradae	Currently undetermined	
Theileria annae	*Ixodes hexagonus*	Camacho *et al*. (2003)
Theileria equi	*Dermacentor variabilis, D. nutalli, Hyalomma* spp , *Boophilus microplus, Rhipicephalus turanicus*	Moltmann *et al*. (1983); Zapf and Schien (1994a); (1994b); Battsetseg *et al*. (2001); Stiller *et al*. (2002)

Table 2.2

Tick vector candidates of the canine piroplasm species.

Babesia infection is generally associated with adult ticks (especially females), however transmission by larval and nymphal ticks has also been documented for *B. canis* (Shortt, 1973). The engorged female tick is the only stage capable of acquiring the infection from the vertebrate host (Friedhoff, 1988). Vertical transmission (transovarial) of *B. canis* is possible and has been demonstrated for *R. sanguineus* (Friedhoff, 1988). This study suggested that *B. canis* may remain infective for five successive generations. Mechanical transmission by most blood-feeding arthropod may also be possible, however limited research has investigated this possibility.

2.4.2 Vertical transmission

Transplacental or perinatal transmission has been known to occur for both *Babesia* and *Theileria* spp (New *et al.*, 1997; Baek *et al.*, 2003). Initial reports of pernatal transmission of parasites *in utero* were noted to occur in humans, suggesting that it was possible for a mother infected with *B. microti*, to transmit the infection to her unborn child (Esernio-Jenssen *et al.*, 1987; New *et al.*, 1997). Further reports have suggested that transplacental transmission of other species such as *Theileria sergenti* (Baek *et al.*, 2003) and *Theileria equi* (Phipps and Otter, 2004) can occur.

Limited study has assessed transplacental transmission of the canine piroplasms, with most information being anecdotal. *Babesia gibsoni* has been found in the blood of young puppies and in their dams, suggesting that transplacental transmission is the most likely cause of infection (Harvey *et al.*, 1988) and a recent study proved this mode of transmission experimentally (Fukomoto *et al.*, 2005a).

2.4.3 Blood transfusion

The role of blood transfusion in the transmission of blood-borne pathogens has become increasingly recognised in both human and veterinary medicine (Herwaldt *et al.*, 2002; Kjemtrup *et al.*, 2002; Powell and Grima, 2002; Cable and Leiby, 2003; Leiby and Gill,

2004). Transfusion babesiosis was first reported in the USA, when a patient received blood infected with *B. microti* (Wittner *et al.*, 1982). The results of a later study indicated that *B. microti* parasites can remain infective under normal blood banking conditions (Eberhard *et al.*, 1995), highlighting the need to screen potential blood donors.

Transfusion-associated transmission has also been reported for at least two species of canine *Babesia*, highlighting the need to screen potential blood donor dogs (Wardrop *et al.*, 2005). *Babesia gibsoni* has been reported to be transmitted during a whole blood transfusion, with the donor blood originating from an American Pit Bull Terrier (Stegeman *et al.*, 2003). Likewise, transfusion-associated transmission has been noted in *B. canis rossi* infections (Jacobson and Clark, 1994). While appropriate screening for *Babesia* and *Theileria* in potential blood donor dogs should be carried out, it has also been reported that the treatment of donor blood with INACTINE PEN110 is highly effective in eradicating *B. microti* from human erythrocytes (Zavizion *et al.*, 2004). It is possible that chemical treatment of *Babesia* infected donor blood from dogs may be also be effective but requires investigation.

2.4.4 Direct blood-to-blood transmission

The possibility of direct blood-to-blood transmission of piroplasms has also been suggested when dogs attack and bite one another. The greatest implication of this form of transmission has been reported in breeds used in dog fighting. A high prevalence of *B. gibsoni* has been described in American Pit Bull Terriers in the USA (Birkenheuer *et al.*, 1999; Macintire *et al.*, 2002; Birkenheuer *et al.*, 2003b) and also in Tosa dogs in Japan (Matsuu *et al.*, 2004a). In both countries, it has been postulated that direct blood-to-blood transmission of *B. gibsoni* may occur during biting or fighting between dogs. Matsuu *et al.* (2004a) also speculated that transmission of the parasite may occur during mating.

2.4.5 Movement of dogs and ticks from areas of endemicity

A major contributing factor in the increased distribution of canine piroplasm species is the movement of family-owned and military working dogs between countries (Anderson *et al.*, 1980; Shaw *et al.*, 2001b). The translocation of chronically infected animals into disease-free areas has previously been suggested as being of significant importance in the spread of *B. gibsoni* in the USA and it is also theorised that military dogs returning from Japan were responsible for the original introduction of this parasite into the US (Anderson *et al.*, 1980).

International travel of dogs has increased recently, with programs such as the Pet Travel Scheme (PETS) contributing to the movement of dogs between countries in Europe (Shaw *et al.*, 2003). Selective analysis of dogs entering the UK revealed many were infected with exotic pathogens including both *B. gibsoni* and *B. canis canis*. This highlights the need to increase surveillance of dogs entering countries know to be free from piroplasm infection to avoid the establishment of these diseases in new parts of the world.

2.4.6 Wild Caniidae species as reservoirs for piroplasms

An important feature of piroplasm spp. infection is the facilitation of wild canids as reservoirs of these parasites. Multiple canine species have been described as potential hosts for canine *Babesia* throughout many regions of the world. Jackals (*Canis aureus*) in India, foxes (*Vulpes vulpes niloticus*), jackals (*Canis aureus lupaster*) and a fenec (*Fennecus zerda*) in Egypt (Maronpot and Guindy, 1970; Botros *et al.,* 1975) and coyotes (*Canis latrans*) in the USA (Yamane *et al.,* 1994) have all been suggested as reservoirs of *B. gibsoni* infection. Notably, coyotes that were experimentally infected with *B. gibsoni* exhibited only mild clinical signs (Roher, 1985), suggesting that they may act as carrier animals. Cape hunting dogs (*Lycaon pictus*) and silver-backed jackals (*Canis mesomelas*) have been associated with *B. canis* infection (Kuttler, 1988). Additionally, *B. canis rossi* was found in the blood of side-striped jackals (*Canis adustus*) in southern Africa (Lewis *et al.*, 1996) and

T. annae has been identified in red foxes (*Vulpes vulpes*) in Spain (Criado-Fornelio *et al.,* 2003a) and the USA (Goethert and Telford, 2003).

Wild canines in Australia, most notably dingoes (*C. familiaris dingo*) have been previously reported with babesiosis (Callow, 1984) and were probably infected with *B. canis vogeli* (Irwin and Hutchinson, 1991). Dingo populations may therefore also represent a potential reservoir for *B. canis vogeli* in Australia

2.4.7 Other mammal species as canine piroplasms reservoirs

It has increasingly been reported that many piroplasm species are not host specific and may be cable of infecting multiple host species (Criado-Fornelio *et al.*, 2003a; Criado-Fornelio *et al.*, 2003c). Such reports have also been published for some of the canine piroplasm species. *Theileria annae* has been found to infect cats and *B. canis canis* has been detected in the blood of both cats and horses (Criado-Fornelio *et al.,* 2003a). *Theileria annae*-like piroplasms have also been identified in skunks and racoons (Goethert and Telford, 2003; Kawabuchi *et al.*, 2005). Other carnivores may also harbour species of piroplasm, potentially capable of infecting dogs such as *Babesia missirolii* and an unnamed piroplasm species identified in badgers (*Meles meles*) (Peirce, 1974; Simsek *et al.*, 2003), *Babesia mephitis* from the striped skunk (*Mephitis mephitis*) (Holbrook and Frerichs, 1970) and *Babesia heischi* and *Babesia hoarei* from Peter's pigmy mongoose (*Helogale undulata rufula*) (Grewal, 1957). Each of these species have never been genetically characterised. Badgers are reported to be commonly infected with the tick *Ixodes hexagonus*, also the presumed vector of *T. annae*. Further research is therefore warranted to determine whether the badger piroplasm species described by Peirce (1974) is actually *T. annae.*

2.5 Life cycles of the Piroplasmida spp.

The life cycle of canine piroplasms is characteristic of that of all apicomplexan parasites in that it generally involves at least three phases of reproduction; gamogony, sporogony and

schizogony (Homer *et al.,* 2000; Kjemtrup and Conrad, 2000). Schizogony occurs within the vertebrate host and the stages gamogany and sporogany occur within the tick vector. Some variation in lifecycle characteristics does exist between members of the *Babesia* and the *Theileria.*

Detailed studies have determined many stages within the lifecycle of both *B. canis* and *B. gibsoni*, however no lifecycle stages have been determined for *Babesia* sp (North Carolina). No detailed observations have been reported for the lifecycle stage characteristics for any of the canine Theilerid /Prototheilerid group species (*T annae*, *B. conradae*) except for *T. equi* (Mehlhorn and Schein, 1998). It can only be assumed that the lifecycle of these species is similar to other *Theileria* and further research is necessary to determine species-specific life cycle stages.

2.5.1 Babesia

i) Stages in the tick vector

The life cycle of *B. canis* is shown in Figure 2.4. Detailed observations of the development of *B. canis* within the gut of the adult tick *Dermacentor reticulatus* have been recorded (Shortt, 1973, Mehlhorn *et al.,* 1980). In addition, comprehensive studies have been carried out on the development of *B. gibsoni* within the midgut of both the larval and nymphal stages of the tick *R. sanguineus* (Higuchi *et al.,* 1999a, Higuchi *et al.,* 1999b). Development is similar for both *B. canis* and *B. gibsoni* and involves the sexual reproductive stage of the life cycle. Merozoites, and trophozoites within canine erythrocytes, are ingested by the tick vector and are microscopically detectable in the gut of the tick about 10 hours after feeding commences (Homer *et al.*, 2000). The trophozoites develop into gametocytes and begin to form a strahlenkörper (ray body) at the anterior of the piroplasm. These in turn form gametes and fuse to produce a zygote, which enters the gut epithelium cells. At this stage, the zygote becomes a kinete which migrates to the salivary glands via the haemolymph (Mehlhorn and Schein, 1984). Kinetes can also enter the eggs of the tick, allowing for transovarial

transmission (Homer *et al*., 2000). Sporogony or the formation of sporozoites occurs within the salivary gland, with many thousands of sporozoites being produced from each initial kinete.

ii) Stages in the vertebrate host

Transmission of the sporozoites from the tick's salivary glands to the canine host generally occurs 2-3 days after tick attachment (Martinod *et al*., 1985). Once inside the host, the sporozoites become merozoites and invade the erythrocytes by a process of endocytosis and form a parasitophorus vacuole (which later disintegrates) within the cell (Homer *et al*., 2000). The merozoites transform into trophozoites and divide by binary fission into additional merozoites, a stage termed schizogony. The newly formed merozoites lyse the host cell and continue to invade and multiply within other erythrocytes. Some of the trophozoites become gametocytes, reproducing once inside of the tick gut.

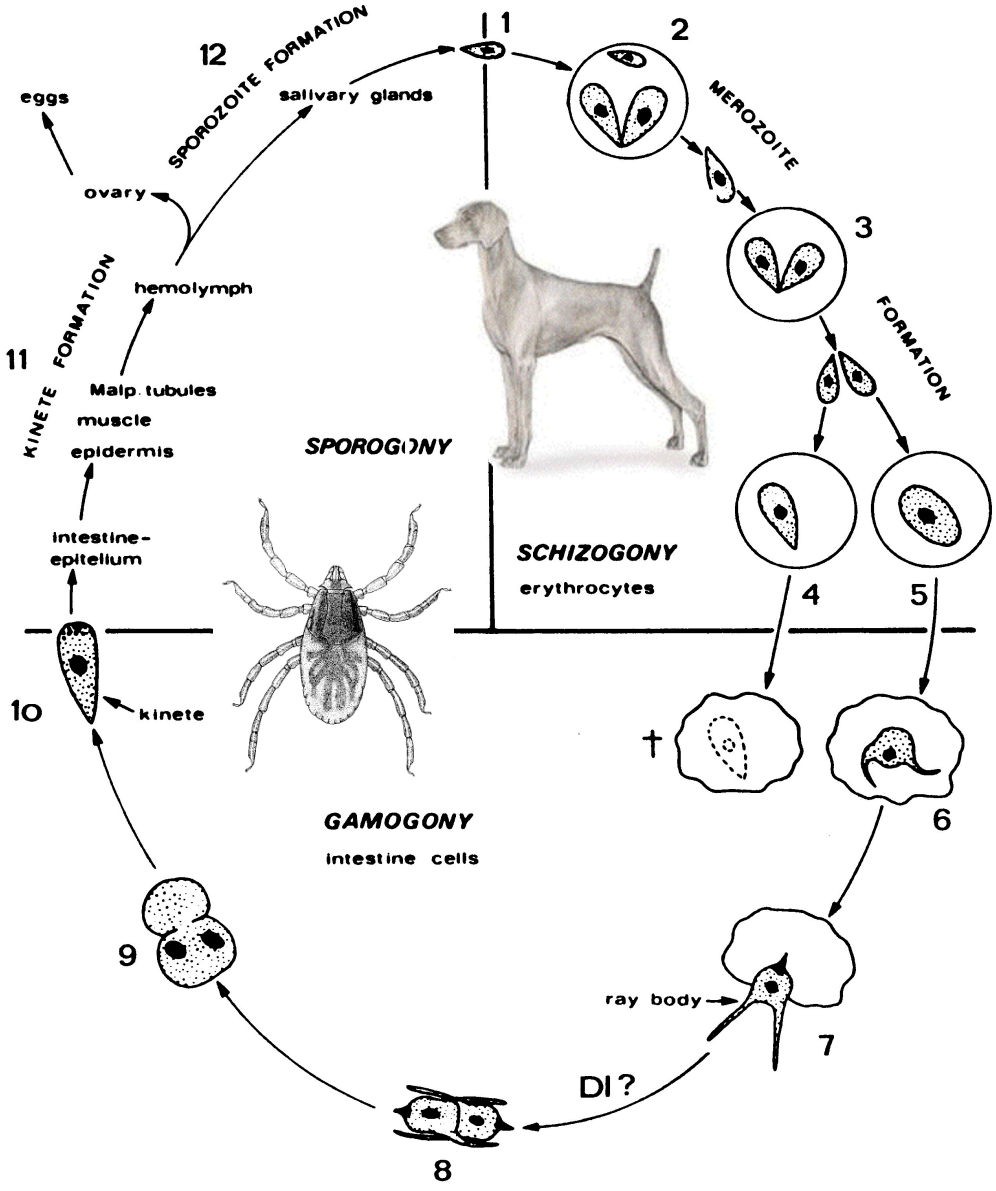


Figure 2.4

Typical three stage life cycle of *Babesia canis*. 1 to 5 – Schizogony within the canine host, 6 to 10 – Gamogany and 11 to 12 – Sporogany in the tick vector (modified from Mehlhorn and Schein, 1984).

2.5.2 Theileria

ii) Stages within the tick vector

Gamogony (the sexual reproductive stage) occurs when infected erythrocytes are ingested by a tick, digested in the gut and allowing for the release of the ovoid stage of *Theileria*. (Mehlhorn and Schein 1984; Kocan 1995). Ovoid stages can then proceed to directly form macrogametes or they form intermediate microgamonts and microgametes stages. Macrogametes then fuse to form zygotes, which in turn develop into motile kinetes. Occasionally, division of the nucleus may begin before kinetes leave the intestinal cells of the vector (Mehlhorn and Schein 1984). Kinetes then migrate into the cells of the tick's salivary gland initiating sporogony (Fawcett *et al*. 1982; Mehlhorn and Schein 1984). Asexual reproduction occurs by growth and nuclear division. This continues, resulting in enlargement of the host cell and its nucleus and the formation of thousands of sporozoites (Mehlhorn and Schein 1984).

ii) Stages within the vertebrate host

The vertebrate stage of the *Theileria* lifecycle is initiated with the sporozoite stage, found in the saliva of a feeding tick (Figure 2.5). The tick then attaches to a suitable host and allows for the transmission of parasites during feeding. Schizogony immediately follows after the parasites have been injected into the host by a feeding tick (Mehlhorn and Schein 1984). A significant difference from the lifecycle of the *Babesia* is the presence of an exoerythrocytic or lymphocytic stage. Non-motile sporozoites come into contact with lymphocytes and enter by a process known as 'zippering' and forming merozoites (Shaw, 2003). Merozoites are then released into the blood stream where they penetrate erythrocytes, undergo binary fission and form the resultant ovoid stage (Mehlhorn and Schein 1984).

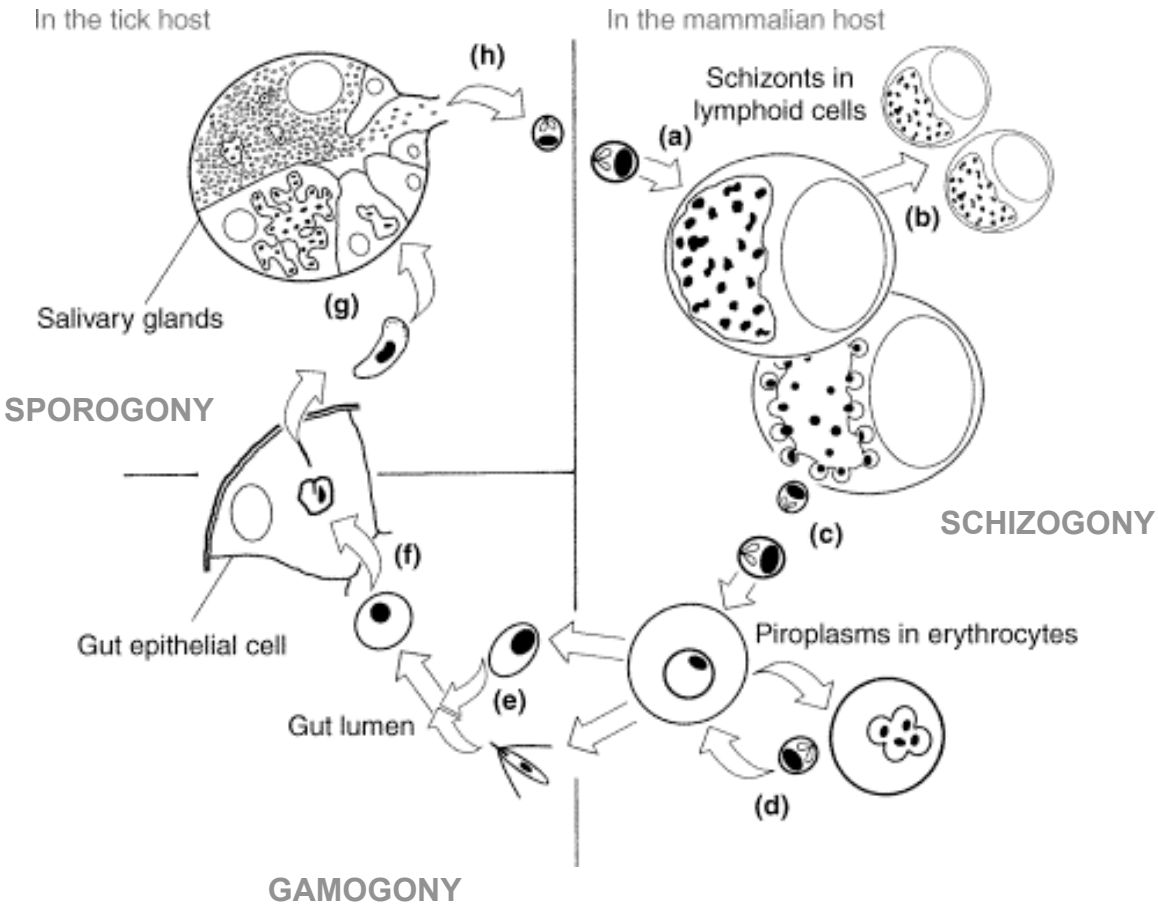


Figure 2.5

Typical three stage life cycle of *Theileria* (Adapted from Shaw, 2003). Sporozoites develop into multinucleate syncytial schizonts (a). Parasite induced host cell proliferation (b). Schizonts differentiate into merozoites which invade erythrocytes (c). Asexual division within the erythrocyte (d). Ticks ingest infected erythrocytes and the formation of gametes and feritilization occurs in the gut lumen (e). Zygotes penetrate the gut epithelial cells and develop into a motile kinete (f). The motile kinete invades the salivary glands (g) and develops into sporozoites, which are then released into the bloodstream of the mammalian host during feeding (h).

2.6 Distribution

The distribution of each of the canine Piroplasmida species is variable with some showing an ever emerging, worldwide dispersal, while others seem to have a relatively restricted distribution, found in a very limited number of countries. The full extent of the distribution of each of the different species is currently unknown and requires further investigation to appreciate the complete epidemiological situation among these protozoa.

2.6.1 Large canine piroplasm spp.

Of the large canine piroplasms (Figure 2.6), *Babesia canis vogeli* has the greatest known distribution, predominantly in semitropical to tropical areas and has been reported in South and North America, Africa, Australia, Asia, Southern Europe and the Middle East (Uilenberg *et al.*, 1989; Taboada *et al.*, 1992; Carret *et al.*, 1999; Caccio *et al.*, 2002; Jefferies *et al.*, 2003). *Babesia canis rossi* is believed to have the most confined distribution, found only in southern Africa (Uilenberg *et al.*, 1989; Carret *et al.*, 1999) and Sudan (Oyamada *et al.*, 2005). *Babesia canis canis* has been reported in France, Hungary (Földvari *et al.*, 2005), The Netherlands (Zandvliet *et al.*, 2004), Slovenia (Duh *et al.*, 2004), Russia (Rar *et al.*, 2004), Switzerland (Casati *et al.*, 2004) Poland and Croatia (Caccio *et al.*, 2002).

The current distribution of the unnamed large *Babesia* sp. is unknown and has only been found within one dog in North Carolina, USA (Birkenheuer *et al.*, 2004b).

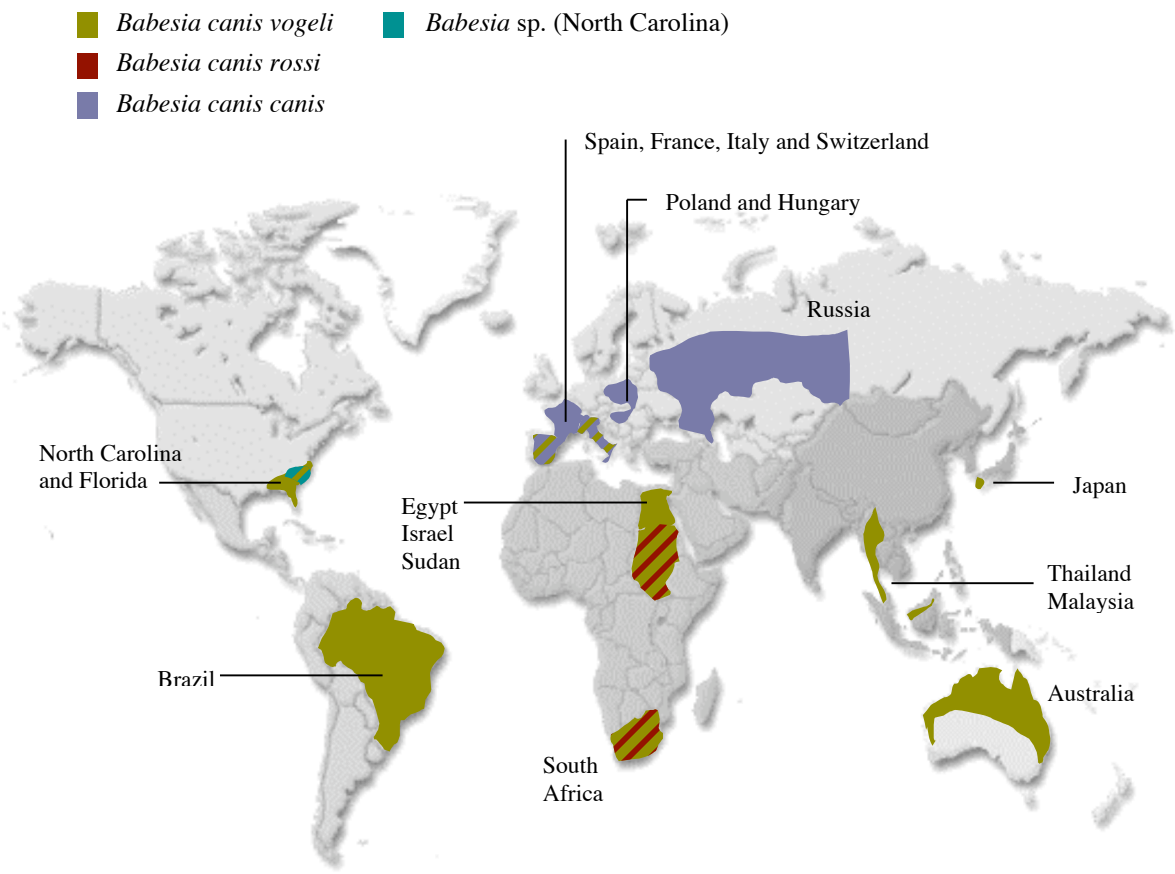


Figure 2.6

Current reported worldwide distribution of the 'large' canine Piroplasmida species

2.6.2 Small canine piroplasm spp.

The current reported distribution of the small canine piroplasms is shown in Figure 2.7. *Babesia gibsoni* has a wide distribution, found in India (Patton, 1910), Japan, Malaysia, Sri Lanka (Zahler *et al.*, 2000b) Korea (Scott *et al.*, 1971; Song *et al.*, 2004), North America (Anderson *et al.*, 1979; Birkenheuer *et al.*, 1999), Italy (Casapulla *et al.*, 1998) Spain (Criado-Fornelio *et al.*, 2003c), France (Zahler *et al.*, 2000a; Suarez *et al.*, 2001), Egypt, Nigeria and Mali (Yamane *et al.*, 1993) and Australia (Muhlnickel *et al.*, 2002). *Theileria annae* has been reported in northern eastern Spain, Portugal (Zahler *et al.*, 2000a; Camacho

et al., 2002) and Massachusetts in the USA (Goethert and Telford, 2003), while *B. conradae* has only been reported in California, USA. A recent report described the presence of small piroplasms in dogs in Hungary (Farkas *et al*., 2004), however the species has yet to be confirmed.

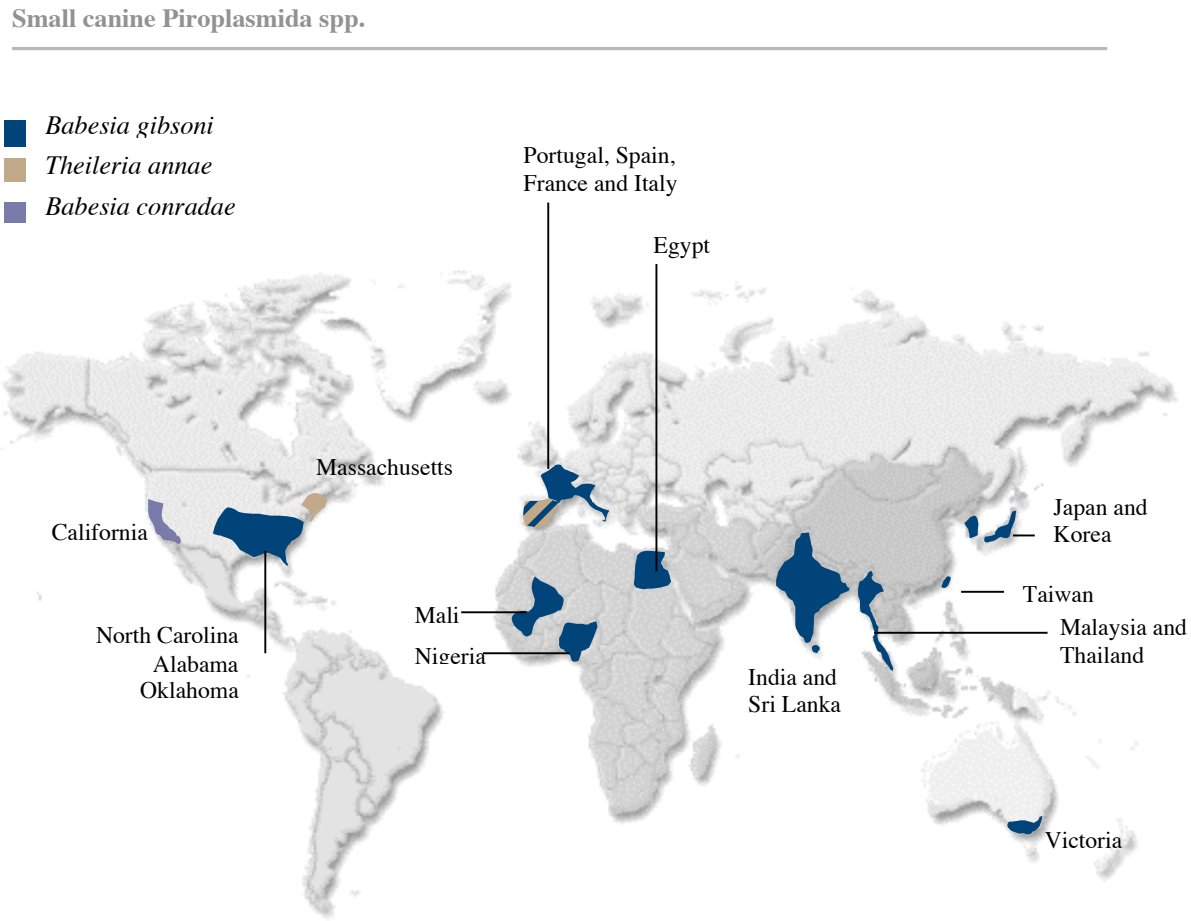


Figure 2.7

Current reported distribution of the 'small' canine Piroplasmida species

2.7 Clinical Signs and Pathogenesis

Dogs suffering from *Babesia* infections have been shown to present with variable clinical signs including, pale mucous membranes, depression, anorexia and jaundice (Irwin and Hutchinson, 1991). Canine babesiosis is generally characterised by haemolytic anaemia and thrombocytopenia a result of direct and indirect (immune-mediated) blood cell damage

induced by the parasites. Additional complications of the disease are variable depending upon the strain and species of *Babesia* involved. Less virulent strains produce a more transient disease while those that exhibit an increased virulence can produce multiple organ dysfunction, which can lead to death of the infected host (Lobetti, 1998; Boozer and Macintire, 2003). Babesiosis can generally be classified as acute, chronic or subclinical (Breitschwerdt *et al*., 1984).

It is also suggested in the literature on the Piroplasmida species that recrudescence of infections is possible (Bronsdon *et al*., 1999), a feature similarly reported in certain *Plasmodium* infections (Mackintosh *et al*., 2004). Recrudescence, or the re-emergence of clinical infection in animals previously known to be infected with a pathogen, is often induced by increased stress levels in the host or by immuno-compromisation. This highlights the possibility that Piroplasmida species can remain inactive within certain organ systems, while not being present in the circulatory system (Ilhan *et al*., 1998). Studies have speculated that inactive piroplasm may exist within the spleen, liver, kidneys or brain of the host, producing no illness for months and even years (Dao *et al*., 1996; O'Connor *et al*., 1999).

2.7.1 Babesia canis subspecies

The acute phase of the infection of all three subspecies is characterised by haemolytic anemia. Acute renal failure, cerebral babesiosis, coagulopathy, icterus, hepatopathy, immune-mediated haemolytic anaemia, acute respiratory distress syndrome and shock have been reported as complications associated with *B. canis* infection (Lobetti, 1998). Each of the *B. canis* subspecies have been reported to produce different disease syndromes (Irwin and Hutchinson, 1991; Schetters *et al*., 1997b). The most virulent subspecies is *B. canis rossi*, characterized by a high proliferation rate. Schetters *et al*. (1997b) reported parasitemia rates that were greater than 1% and that the level of parasitemia showed a correlation with the degree of haemolysis and haemoglobinuria. Hypoglycemia and icterus are also associated with infection (Keller *et al*. 2004). *Babesia canis rossi* can also produce mild

infections (Malherbe *et al.*, 1976; Moore and Williams, 1979; Reyers *et al.*, 1998). Such non-anaemic babesiosis has been reported to be associated with severe azotemia, electrolyte and acid-base disturbances and sometimes leukopenia (Reyers *et al.*, 1998). Differences in virulence exhibited between cases of *B. canis rossi* infection in South Africa may relate to the potential of co-infections with *B. canis vogeli* (Matjila *et al.*, 2004).

Babesia canis canis exhibits a lesser virulence and comparative studies with *B. canis rossi* concluded each of the two subspecies produced a different disease syndrome (Schetters *et al.,* 1997b). Clinical disease resulting from *B. canis canis* infection was correlated to changes in the dog's coagulation system and not the level of parasitemia. *Babesia canis canis* may proliferate in deep tissues and also shows evidence of autoagglutination (Schetters and Montenegro-James, 1995). Fatal cases have also been reported (Matjila *et al.*, 2005). *Babesia canis vogeli* has been shown to be the least virulent of the three subspecies, with the acute phase of the disease most notable in pups (Irwin and Hutchinson, 1991). Co-infections of *B. canis canis* and *B. canis vogeli* may also occur (Caccio *et al.*, 2002; Duh *et al.*, 2004), further complicating the disease pathogenesis.

2.7.2 Babesia gibsoni

Inokuma *et al.* (2005) reported that *B. gibsoni* infections with low-level parasitaemia produce clinical and laboratory findings similar to those exhibited by immune mediated haemolytic anaemia. This similarity can lead to misdiagnosis and has also been reported by Muhlnickel *et al.* (2002), when a case of *B. gibsoni* infection in Australia was initially misdiagnosed as immune mediated haemolytic anaemia. It has also been suggested that macrophages phagocytose both parasitised and non-parasitised erythrocytes, causing extravascular haemolysis and splenomegaly (Murase *et al.,* 1996). This signified that oxidative damage within the erythrocytes, including those not parasitised, is a result of *B. gibsoni* proliferation. *Babesia gibsoni* parasites have also been shown to preferentially infect and multiply in younger erythrocytes (Murase *et al.,* 1993).

2.7.3 Babesia conradae

Wozniak *et al.* (1997) demonstrated that haemolytic, regenerative anaemia occurs within all *B. conradae* (described as *B. gibsoni*) infections. It was suggested that the destruction of erythrocytes was a result of mechanical disruption of the cells by the infecting parasites, complement-dependent immune-mediated erythrolysis and the phagocytosis of antigenically altered or opsonized erythrocytes. Hepatic lesions are another distinctive feature of the infection, characterised by hepatitis, hepatocellular atrophy, perivenular fibrosis and Kupffer cell hypertrophy (Wozniak *et al.,* 1997). Additionally, vasculitis and glomerulonephritis have been reported and are believed to be a consequence of the immune mediated component of the disease (Wozniak *et al.,* 1997).

2.7.4 Theileria annae

Intense anaemia, azotemia and thrombocytopenia, with limited leucocytosis and renal dysfunction and sometimes death have been reported in *T. annae* infected dogs in north-west Spain (Camacho *et al.*, 2001; Guitian *et al.*, 2003; Camacho *et al.*, 2004). In addition, a splenectomized dog infected with *T. annae* presented with hypothermia, trembling and dark urine and clinical signs included pale mucous membranes, tachycardia, tachypnea and lymphadenopathy (Camacho *et al.*, 2002).

2.8 Detection and diagnosis of canine piroplasm infections

Effective diagnosis of *Babesia* infections is important in their monitoring, management and control (McLaughlin *et al.*, 1992). A large diversity of diagnostic techniques exist, each of which has its own limitations. The diagnostic tests for piroplasmosis can be divided into three broad categories; traditional methods, including microscopy and culture; serological techniques, and molecular-based methods. It is increasingly recognised that a combination of detection techniques is necessary for accurate diagnosis. Limitations of clinical data, parasite morphology and serological cross-reactivity, have lead to an increased interest in molecular based methods and highlights the need for their application in clinical medicine.

2.8.1 Light Microscopy

The most widely used technique for the detection of *Babesia* is the examination of thin blood smears stained with either Wright or Giemsa stain (Homer *et al.,* 2000). Erythrocytes are scanned for the presence of piroplasms. Parasitaemia levels have been found to be concentrated in blood taken from ear-tip capillaries (Breitschwerdt, 1984), therefore using ear-tip blood smears may increase the likelihood of detecting piroplasm infections. The sensitivity of microscopy is suggested to be one parasite per 10^5 erythrocytes (Bose *et al.,* 1995). This technique is limited in that morphologically similar species cannot be distinguished (Conrad *et al.,* 1992) and accurate diagnosis is dependent on the experience of the microscopist (Morgan, 2000).

2.8.2 Serological Tests

Multiple immunodiagnostic techniques have been created to detect antibodies to *Babesia spp*. (reviewed by Bose *et al*., 1995). The two that are routinely applied to the diagnosis of *Babesia* infections in dogs are the immunofluorescent antibody test and the enzyme-linked immunosorbent assay.

i) Immunofluorescent Antibody Test (IFAT)

This test is a commonly used method of diagnosing *Babesia* and *Theileria* infections by detecting the presence of antibodies to the parasites within the host serum. The test uses antigen, in the form of parasite-infected blood applied to glass slides, host serum titrated to various dilutions and fluorescein-labelled antibodies. The serum and antibodies are added to the antigen, incubated and analysed using fluorescent microscopy.

Immunofluorescent Antibody Tests have been developed for both *B. canis* and *B. gibsoni*, however limitations have been suggested to exist for such methodology. Levy *et al.* (1987) found that 3.8% of dogs analysed in North Carolina were seropositive for *B. canis* using IFAT, however recognised the possibility of cross-reactivity with *B. gibsoni*. Later, the

seroprevalence of *B. canis* in greyhounds from Florida was determined using IFA screening and was reported to be 46% (Taboada *et al.*, 1992). A *B.canis* seropositive dog has been reported to have an antibody titre cut-off of ≥ 1:80 (Levy *et al.*, 1987; Taboada *et al.*, 1992). IFAT was first developed for the diagnosis of *B. gibsoni* infections by Anderson *et al.* (1980), who set the seropositive cut-off titre at >1:64. The use of IFAT for the diagnosis of *B. gibsoni* was later evaluated and the need for optimal cut-off titres to be established to avoid false-positive results due to antigen cross-reactivity was described. The IFAT for *B. gibsoni* was found to be cross-reactive with *B. canis, Toxoplasma gondii* and *Neospora caninum* (Yamane *et al.*, 1993). It is also reported that dogs that are acutely infected with *Babesia* may be seronegative (Breitschwerdt *et al.,* 1983) and it is also difficult to assess whether the dog currently has an infection or has previously been infected.

ii) Enzyme-linked immunosorbent assay (ELISA)

The ELISA was first applied to *Babesia* diagnosis in the detection of *B. bovis* and *B. caballi* in cattle by utilizing antigens from infected erythrocytes. The earliest application of the ELISA to canine *Babesia* was by Martinod *et al.* (1985). Their study developed the assay to detect antibodies against *B. canis,* in addition to antibodies to the vectors *D. reticulatus* and *I. ricinus.* The ELISA is limited by *Babesia* strain differences eliciting different antibody responses and producing variable seroreactivity (Reiter and Weiland, 1989). Verdida *et al.* (2004) developed an improved ELISA using recombinant truncated P50 surface antigen for the serodiagnosis of *B. gibsoni* infection.

2.8.3 Polymerase chain reaction

The advent of the polymerase chain reaction (PCR) has shown significant promise in the detection of pathogens and the diagnosis of disease over the past decade. PCR is a relatively new molecular procedure that was first described in 1985 (Saiki *et al.*, 1985; Mullis, 1990) and involves the *in vitro* amplification of target nucleic acid sequences by primer directed DNA synthesis. Initial use of PCR as a detection technique for *Babesia* was demonstrated in

non-canine species, most notably *B. bovis, B. microti* and *B. bigemina* and was shown to have a significant degree of sensitivity and specificity (Fahrimal *et al.,* 1992, Persing *et al.,* 1992 and Figueroa *et al.,* 1992). Since then, the technique has been applied to many other species of *Babesia*.

i) PCR detection of Babesia DNA in canine blood

PCR application to canine *Babesia* was first demonstrated on *B. canis*, involving DNA amplification for sequencing and phylogenetic comparison (Allsopp *et al.,* 1994). Later studies have shown the ability of PCR to be a useful diagnostic tool for the detection and phylogenetic analysis of the canine *Babesia* species. A majority of these studies used amplification of partial regions of the small subunit ribosomal RNA gene as the basis of diagnosis (Carret *et al.,* 1999). The small subunit ribosomal RNA gene is useful in that it is a highly conserved gene, showing limited nucleotide sequence variation. The gene exhibits a steady accumulation of mutations on an evolutionary scale and is therefore valuable in distinguishing different species (Hillis and Dixon, 1991). Different regions of the small subunit ribosomal RNA gene have been amplified by PCR, including the 18S rRNA gene (Conrad *et al.,* 1992; Allsopp *et al.,* 1994; Kordick *et al*., 1999; Zahler *et al.,* 2000b; Zahler *et al.,* 2000c; Kjemtrup *et al*., 2000a; Ano *et al.,* 2001; Birkenheuer *et al*., 2003a), the first and second transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene for *B. canis* (Zahler *et al.,* 1998).

A partial region of the β-tubulin gene has also shown promise in PCR diagnosis (Caccio *et al.,* 2000). The gene contains an intron that is extensively variable in length and sequence among species of *Babesia* and *Theileria*. Species could be differentiated on the basis of the size of the PCR product. This technique has as yet, not been applied to the canine *Babesia*. Additionally, the genetic sequences of the heat shock-related proteins 70 and 90, show promise as PCR target regions as they are highly conserved (Muhlschlegel *et al*., 1995). Of

the *Babesia* species, amplification of the heat shock protein genes has been applied to *B. bovis*, *B. microti* (Ruef *et al*., 2000) and *B. gibsoni* (Yamasaki *et al*., 2002).

ii) PCR detection of Babesia DNA in Ticks

PCR has also been applied to the detection of pathogen DNA within tick vectors and has been extensively reviewed by Sparagano *et al*. (1999). *Babesia bigemina* and *B. bovis* (Sparagano *et al*., 1999) and *B. caballi* and *B. equi* (Battsetseg *et al*., 2001) have been detected using PCR but its application to the canine *Babesia* has not been reported to date. The main problem associated with PCR analysis on ticks is contamination by non-target organisms on the surface of the ticks, which can be overcome by ethanol sterilization (Sparagano *et al*., 1999).

iii) Specificity and detection limits of PCR

A superior feature of PCR as a diagnostic tool is its high detection limit and specificity. Primers can be designed to be genus specific or can amplify species-specific sequences of DNA, allowing for detection of a single species. Assessment of PCR sensitivity for the detection of canine *Babesia* has been carried out by serially diluting blood samples of a known percentage parasitaemia (Ano *et al.,* 2001, Fukumoto *et al*., 2001; Birkenheuer *et al*., 2003a; Jefferies *et al*., 2003). The tests were shown to detect parasitaemias ranging from 0.000118 to 0.00000073 %. Caution is suggested in interpreting detection limit calculating using serially diluted blood due to likely variations in erythrocyte levels in the host (Birkenheuer *et al*., 2003a). The high degree of sensitivity of PCR is important in effectively diagnosing acute infections when the parasitaemia is low (>1%). PCR has been found to be more sensitive than blood smear examination and IFAT for the diagnosis of acute *Babesia* infections (Krause *et al*., 1996).

High levels of sensitivity can also be considered a downfall of PCR as it can produce false positives due to nucleic acid contamination (Persing, 1991). The use of ultra-violet

irradiation of reagents and primers has been shown to be successful in reducing and even removing all PCR reagent contamination (Sarkar and Sommer, 1990), however this only offers a treatment to the problem and fails to offer a preventative solution. Contamination is best controlled by stringent execution of good laboratory practice, including the physical separation of pre and post amplification procedures, design of species specific primers and ultra-violet irradiation of laboratory equipment (Persing, 1991).

2.8.4 PCR-Restriction fragment length polymorphism (RFLP)

The use of RFLP allows for the discrimination of amplified DNA products on the basis of nucleotide differences. Restriction enzymes are used to cleave DNA at specific sites, producing a series of smaller DNA fragments that can be used as a means of differentiating species and/ or genotypes. The advantage of this method is that amplified DNA does not need to be sequenced, reducing the time and cost of detection and differentiation. The amplified ITS1, ITS2 and the 5.8S rRNA were subjected to restriction-fragment-length polymorphism analysis and provided the basis for an effective means of discriminating between the three subspecies of *B. canis* (Zahler *et al*., 1998). Each of the *B. canis* subspecies have also been differentiated by RFLP using a partial region of the 18S rRNA gene (Carret *et al*., 1999).

2.8.5 Quantitative PCR

As separate from traditional PCR, quantitative PCR (Q-PCR) allows for the estimation of the initial concentration of target DNA within a sample using various fluorescence technologies. The use of Q-PCR for the detection and quantification of piroplasms was initially developed for *Theileria sergenti* using TaqMan chemistry (Jeong *et al*., 2003). The TaqMan Q-PCR was reported to detect a parasitaemia of 0.00005%, making it highly useful in detecting chronic infection and also in the effective determination of parasitaemia status in cattle. Q-PCR has not yet been reported for the detection of the canine piroplasm spp.

2.8.6 Loop-mediated isothermal amplification method (LAMP)

Loop-mediated isothermal amplification was first described by Notomi *et al*. (2000) and is a method that allows for the amplification of DNA with high levels of specificity, efficiency and rapidity under isothermal conditions. Using four primers that recognise six distinct regions on the target DNA and DNA polymerase, multiple stem-loop DNA structures are synthesized. In less than an hour, the cycling reaction can produce 10^9 copies of the target region of DNA (Notomi *et al*., 2000). Ikadai *et al*. (2004) developed a LAMP assay for the detection of *B. gibsoni*. One of the most significant advantages of this method is the time requirement, with the LAMP reaction time limited to one hour, while PCR can take up to four hours (Ikadai *et al*., 2004).

2.8.7 Filter paper-based DNA detection

The use of filter paper for the storage and archiving of DNA samples for subsequent DNA amplification was first developed by Belgrader *et al*. (1995). A number of commercial filter papers including Isocode Stix and Whatman® FTA cards were later developed as a methods of collection, shipment, archiving and purification of DNA from blood and tissue samples for PCR analysis. FTA treated filter paper contains protein denaturants, chelating agents and a free radical trap designed to enable the protection and long term binding of the DNA to the filter matrix (Belgrader *et al*., 1995). Other substances within the sample, such as potential PCR inhibitors found in blood, are not bound to the FTA matrix and can be removed during serial washing of the sample. Samples stored on FTA cards show significant archiving potential, with DNA stability shown to exist after greater than four years (Li *et al*., 2004).

The use of filter-based technology has primarily been for forensic applications whereby DNA can be isolated directly from mammalian and plant tissues (Natarajan *et al*., 2000; Ivanov *et al*., 2002; Raina and Dogra, 2002; Smith and Burgoyne, 2004; Harvey, 2005). There has also been increased use of this technique for the PCR amplification of pathogen DNA within a sample, for example, parasite DNA within a human blood sample (Kuboki *et*

al., 2003; Becker *et al*., 2004; Chappius *et al*., 2005). This technique has been used for the detection of *B. microti* DNA (Okabayashi *et al*., 2002) but has not yet been applied to the canine piroplasm species.

2.8.8 Other methods of detection

Other detection techniques that have the potential to be applied to the canine piroplasms include the inoculation of susceptible animals with blood from a suspected case (Krause *et al*., 1996), the hydroethidine-flow cytometry method (Bicalho *et al*., 2004), reverse line blot hybridisation assays (Gubbels *et al*., 1999; Georges *et al*., 2001; Almeria *et al*., 2002; Oura *et al*., 2004) and the latex agglutination test (Xuan *et al*., 2001).

2.9 Prevention and Treatment of Piroplasm Infections

The most successful method for the prevention of babesiosis is to avoid exposure to ticks (Smith and Kakoma, 1989; Homer *et al*., 2000). Transmission of the parasites can also be limited by the removal of ticks within 24 hours of attachment, as there is a direct correlation between attachment time and the transmission of sporozoites (Homer *et al*., 2000).

2.9.1 Acaricide therapy

Synthetic pyrethroids are suggested to be effective tick control compounds for companion animals, having both acaricidal and repellant properties. Two recently developed commercial therapies are a combination of imidacloprid and permethrin (Advantix®; Bayer Healthcare AG, Germany) and combined fipronil and methoprene (Frontline Combo®; Merial, France), each showing high efficacy against ticks on naturally infected dogs (Young *et al*., 2003; Otranto *et al*., 2005). Other acaricidal therapies include collars impregnated with flumethrin and propoxur (Fourie *et al*., 2003). The use of pheromones, kairomones and allomone have also been suggested as tick control agents (Sonenshine, 2004). As the complete eradication of ticks is considered impractical and the continuous application of chemicals not stustainable (Peter *et al*., 2005), other control stategies may need to be considered.

The development of resistance in dogs to the tick *R. sanguineus* has been repetitively studied (Theis and Budwiser, 1974; Bechara *et al.*, 1994). While most research has suggested the absence of induced resistance, one study reported the possibility of resistance, warranting further investigation (Inokuma *et al.*, 1997). Vaccination may also be effective against tick attachment. A vaccine based on the use of a recombinant gut antigen has been developed against *Boophilus microplus*, a cattle tick and vector of bovine babesiosis, reducing the number of engorged females and their larvae (Willadsen and Kemp, 1989; Prichard and Tait, 2001). This technique has potential in the development of a vaccine against the tick vectors of the canine *Babesia*.

2.9.2 Drug prophylaxis

Prophylactic chemotherapy for *B. canis* infection has been demonstrated with imidocarb (Vercammen *et al.*, 1996a) and doxycycline (Vercammen *et al.*, 1996b). Imidocarb has previously been shown to give a two-week protection against experimental infection. Doxycycline was proven to offer some protection, however asymptomatic infection could not be ruled out.

2.9.3 Drug treatment

i) Babesia canis

Diminazene aceturate and imidocarb are commonly used babesiacidal drugs (Kuttler, 1988; Jacobson *et al.*, 1996). Imidocarb dipropionate is suggested to be effective against all large *Babesia* species, including *B. canis* and is administered by intramuscular injection, followed by a second dose 14 days later (Kuttler *et al.*, 1975). Other antibabesial drugs used against *B. canis* infection including amicarbalide, euflavine, quinoronium sulfate and chloroquine have been reported however each has shown poor efficacy and adverse side effects (Lobetti, 1998).

Trypan blue is also used to treat dogs presenting with severe shock associated with *B. canis rossi* infection (Boozer and Macintire, 2003; Jacobson *et al.*, 1996). Further supportive treatments for acute renal failure, cerebral babesiosis, immune-mediated haemolytic anaemia, disseminated intravascular coagulation, pulmonary oedema and shock are described by Jacobson and Swan (1995). The use of lipotropic drugs, haematinics, vitamins and glucocorticoids may also aid in the supportive treatment of *B. canis* infection (Jacobson and Swan, 1995).

ii) Babesia gibsoni

Drug treatments for *B. gibsoni* infections have included phenamidine isethionate (Groves and Vanniasingham, 1970), diminazene aceturate (Farwell *et al.*, 1982) and imidocarb (Boozer and Macintire, 2003) however each fail to totally eliminate circulating parasites. Cytidine 5'- monophosphate and inosine 5' – monophosphate may also have an inhibitory effect on the replication of *B. gibsoni* (Hossain *et al.*, 2004). Clindamycin has also been assessed as a treatment for *B. gibsoni* infection (Wulansari *et al.*, 2003). No significant differences in the level of parasitaemia were reported between untreated and treated dogs, however, parasites within the erythrocytes of treated animals showed signs of morphological abnormalities. Clindamycin treatment also resolved anaemia and other clinical manifestations after the acute stage of infection (Wulansari *et al.*, 2003).

Recently, the efficacy of a combined treatment of atovaquone and azithromycin has been assessed (Birkenheuer *et al.*, 2004a). Results suggested that the combined therapy either eliminated or suppressed infections to a limit below detection. Some dogs in this study, did however fail to respond to drug therapy and remained PCR positive for *B. gibsoni* after treatment. Thus, it can be speculated that combined atovaquone and azithromycin may only be effective in some dogs and requires further investigation. The efficacy of atovaquone by itself was also assessed both *in vivo* and *in vitro* (Matsuu *et al.*, 2004b). Although a reduction in parasite numbers was observed in the presence of atovaquone, complete eradication of

infection was not observed *in vivo*. Drug resistance was also demonstrated for atovaquone (Matsuu *et al*., 2004b) and has been found to be associated with mutations within the cytochrome b gene (Birkenheuer and Marr, 2005; Matsuu *et al*., 2005).

Interestingly, the effects of plant extracts on *B. gibsoni* cultured *in vivo* in mice have also be investigated using 45 different plant species from central Kalimantan, Indonesia (Subeki *et al*., 2004). Five of the plant extracts (sourced from *Arcangelisia flava, Curcuma zedoaria, Garcinia benothamiana, Lansium domesticum* and *Peronema canescens*) showed significant anti-babesial activity with IC_{50} values ranging from 5.3 to 49.3 μg/ml. Extracts taken from *A. flava* gave the highest antibabesial activity. Further investigation was performed into the active compounds found within *A. flava* and their effect on *B. gibsoni* in culture (Subeki *et al*., 2005a). In addition, bioassay-guided fractionation of the Indonesian plant *Phyllanthus niruri* identified three possible anti-babesial and anti-malarial compounds (Subeki *et al*., 2005b).

The assessment of anti-babesial activity of plants has also been studied in South Africa, with four ethnoveterinary crude plant extracts being tested against *B. caballi in vitro* (Naidoo *et al*., 2005). *Rhoiscissus tridentata, Elephantorrhiza elephantina, Aloe marlothii* and *Urginea sanguinea* were all assessed, with only *E. elephantina* acetone extracts shown to be effective against *B. caballi* parasites at a concentration of 100 μg/ml. Further study is required to determine the active compounds within such plant extracts to allow for the development of possible anti-babesial drugs.

iii) Other canine piroplasm species

Limited study has investigated potential drug therapies for each of the recently described canine piroplasm species. Treatment of an infection with an unnamed *Babesia* sp. from North Carolina resulted in the resolution of clinical signs (Birkenheuer *et al*., 2004b). Drug therapy with imidocarb dipropionate has also been reported for *T. annae* infections but was

found to be unsuccessful (Camacho *et al.*, 2002). Anti-theilerial drugs include parvoquone, buparvaquone, halofunginone lactate, and parvoquone-plus-furosemide (Njau *et al.* 1985; Mbwambo *et al.* 1987; Mbwambo and Mpokwa 1989; Mbwambo *et al.* 2002) and could potentially be used against *T. annae*.

2.9.2 Protective immunity and vaccination

Dogs that are initially infected with *Babesia* often do not become re-infected due to the effect of protective immunity. Vercammen *et al.* (1997) established that immunity existed for at least 5 months (and even up to 8 months) after an initial *B. canis* infection. No cross-protection between the subspecies of *B. canis* has been observed (Schetters *et al.*, 1995; Vercammen *et al.*, 1997), which suggests antigenic variation exists between the species. Protective immunity is also the basis for an effective vaccine. Multiple vaccines based on soluble parasite antigens have been developed for *B. canis* infections and some are availably commercially (Moreau *et al.*, 1989; Schetters *et al.*, 1995; Schetters *et al.*, 1997a). Immunization of dogs against *B. gibsoni* has also been suggested using recombinant surface antigen P50 (Fukumoto *et al.,* 2005b).

CHAPTER THREE

Review of Literature on *Anaplasma platys* infection of dogs (Canine Infectious Cyclic Thrombocytopenia)

Harvey *et al*. (1978) first described canine infectious cyclic thrombocytopenia (CICT) when *Rickettsia*-like organisms were observed within the platelets of dogs. The causative agent of the disease is now recognised as *Anaplasma platys* (formerly *Ehrlichia platys*, French and Harvey, 1983). CICT generally presents with few clinical signs and is therefore difficult to diagnose. A number of diagnostic methods to detect *A. platys* already exist, although many have their own limitations.

Infection with *A. platys* is considered an emerging disease, although whether this increasing distribution of this pathogen is a reflection of increased awareness in addition to the use of more sensitive detection techniques, rather than a true emergence of disease remains inconclusive.

3.1 Taxonomic classification

Members of the order Rickettsiales and in particular, the families Anaplasmatacea and Rickettsiacea have recently been reorganised, while also unifying and redesignating species belonging to the genera *Ehrlichia, Cowdria, Anaplasma* and *Neorickettsia* (Dumler *et al.*, 2001). This reorganisation of the Rickettsiales, including the abolishment of the tribes Ehrlichieae and Wolbachiae, has received some level of disagreement with Uilenberg *et al.*

(2004) suggesting that *Anaplasma phogocytophila, Anaplasma platys* and *Anaplasma bovis* be re-classified under a new genus.

Anaplasma platys belongs to the family Anaplasmataceae and although initially classified as a member of the genus *Ehrlichia*, this bacterium has now been reclassified on the basis of the 16S rRNA gene and is now recognised as belonging to the genus *Anaplasma* (Dumler *et al.*, 2001).

3.2 Phylogeny and evolutionary relationships of the Anaplasmacae

Anaplasma platys along with other *Anaplasma* species such as *Anaplasma marginale, A. centrale*, *A. ovis* and *A. phagocytophila* form a distinctive phylogenetic clade separate from the genera *Ehrlichia*, *Wolbachia* and *Neorickettsia* on the basis of the 16S rRNA (Figure 3.1) and GroESL genes (Dumler *et al*., 2001; Yu *et al*., 2001; Lee *et al*., 2003). Both the citrate synthase gene (Inokuma *et al*. 2001a) and the rpoB gene (Taillardat-Bisch *et al*. 2003) have also been used for phylogenetic investigation of the Anaplasmacae.

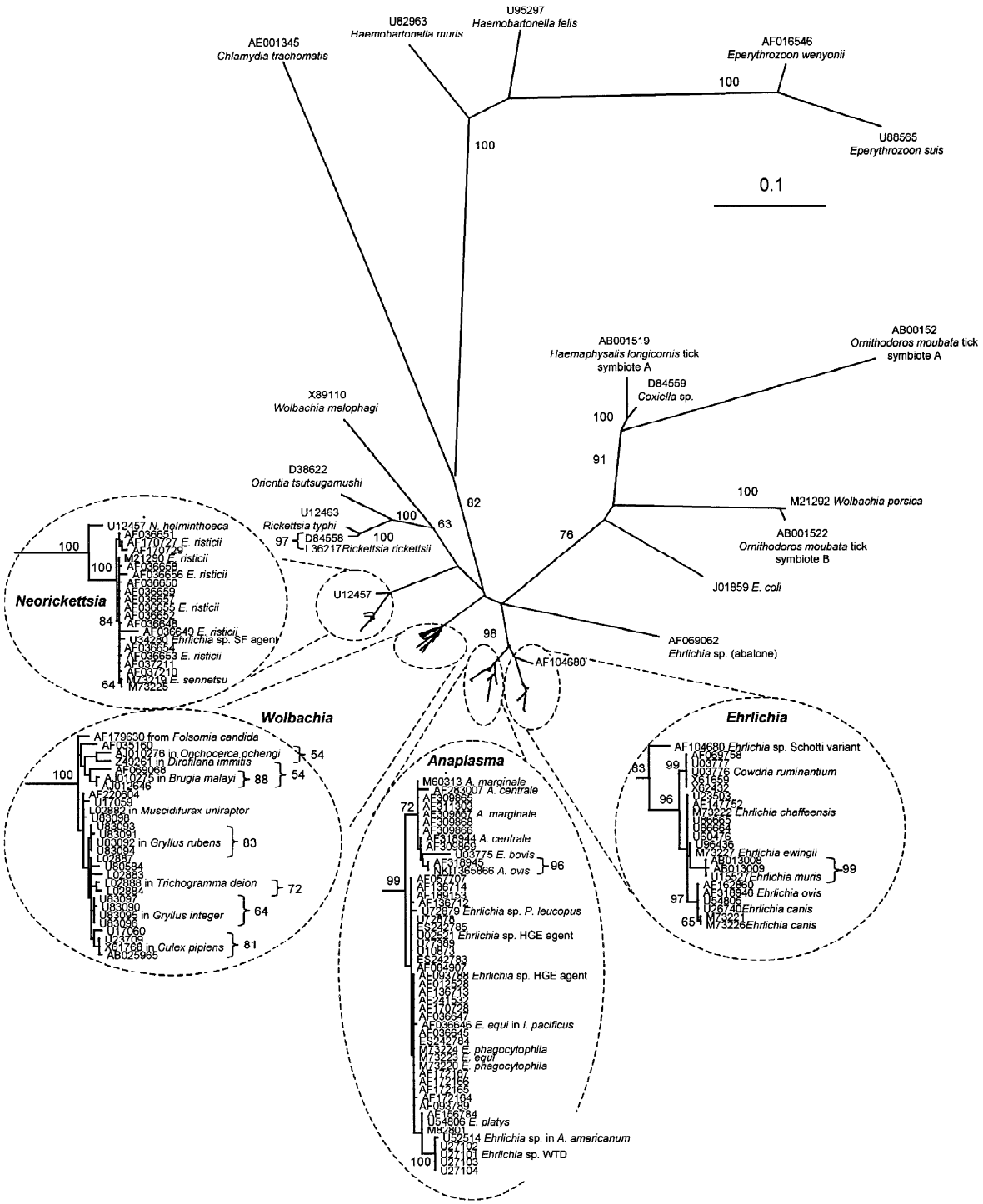


Figure 3.1

Phylogenetic tree based on the small subunit (16S) rRNA gene of *Anaplasma*, *Ehrlichia*, *Neorickettsia* and *Wolbachia* spp. (Dumler *et al.*, 2001)

3.3 Morphology

Morphologically, *A. platys* organisms are similar to the other members of the genera *Ehrlichia* and *Anaplasma*, characterised as small, gram-negative cocci, which may be polymorphic (Rikihisa, 1991). Within the host cell, they appear as basophilic inclusions when stained with Giemsa and may be single organisms or as morulae (Chang *et al*., 1996). Morulae are characterised by multiple organisms clustered together to form a globular mass of bacterial cells and enveloped by the host membrane (Rikihisa, 1991) and may contain as many as 15 rickettsia per host vacuole (Arraga-Alvardo *et al*., 2003).

Organisms range from 0.45 to 1.55 μm in diameter (Arraga-Alvardo *et al*., 2003). Ultrastructural studies using electron microscopy revealed the presence of fine fibrils in the central region of most organisms and appear to be bound by both an inner and outer membrane (Arraga-Alvardo *et al*., 2003, Figure 3.2). Binary fission of some organisms was also observed.

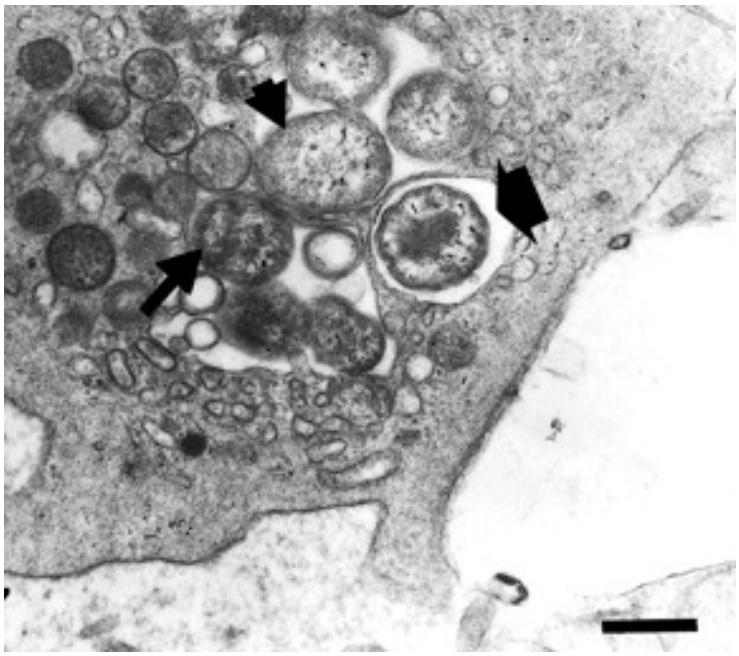


Figure 3.2

Image of *A. platys* morulae within canine platelets (Arraga-Alvardo *et al*., 2003)

3.4 Transmission

Tentative evidence has suggested that the tick *R. sanguineus* is the vector responsible for the transmission of *A. platys* based on geographic distribution, molecular and serological studies (Chang *et al.,* 1996; Inokuma *et al*., 2000; Motoi *et al.,* 2001). Experimental infection of *R. sanguineus* with *A. platys* failed however, and it was suggested that this tick might not act as the vector for *A. platys* (Simpson *et al*., 1991). Further studies need to be carried out to determine whether *R. sanguineus* is actually responsible for *A. platys* transmission.

It has also been suggested that other arthropod species may act as vectors of *A. platys.* Martin *et al*. (2005) have speculated that the louse species, *Heterodoxus spiniger* may act as a vector for *A. platys* in Australia, however it could not be determined whether the *A. platys* DNA in the lice was a reflection of the ingested blood meal or whether this pathogen was actually infecting the louse.

3.5 Life cycle

As the tick vector of *A. platys* has not been confirmed (Simpson *et al*, 1991), there have been no studies on the development of these organisms within the arthropod host. If indeed *R. sanguineus* is responsible for the transmission of *A. platys* it could be inferred that life cycle events within the tick are similar to those of *E. canis* infections. *Ehrlichia* organisms enter the tick midgut while feeding on vertebrate blood, then move into the tick hemocytes and into the salivary glands (Smith *et al*., 1976). The vertebrate host becomes infected during feeding of the tick vector. *Ehrlichia* species multiply by binary fission within both the tick vector and the canine host (Woldehiwet and Ristic, 1993).

3.6 Distribution

Anaplasma platys has an increasingly worldwide distribution (Figure 3.3) and is considered an emerging pathogen (Rikihisa, 2000). As stated previously, whether this is a reflection of

increased awareness and greater specificity of detection methods or a true expansion of the distribution and prevalence of this disease remains inconclusive.

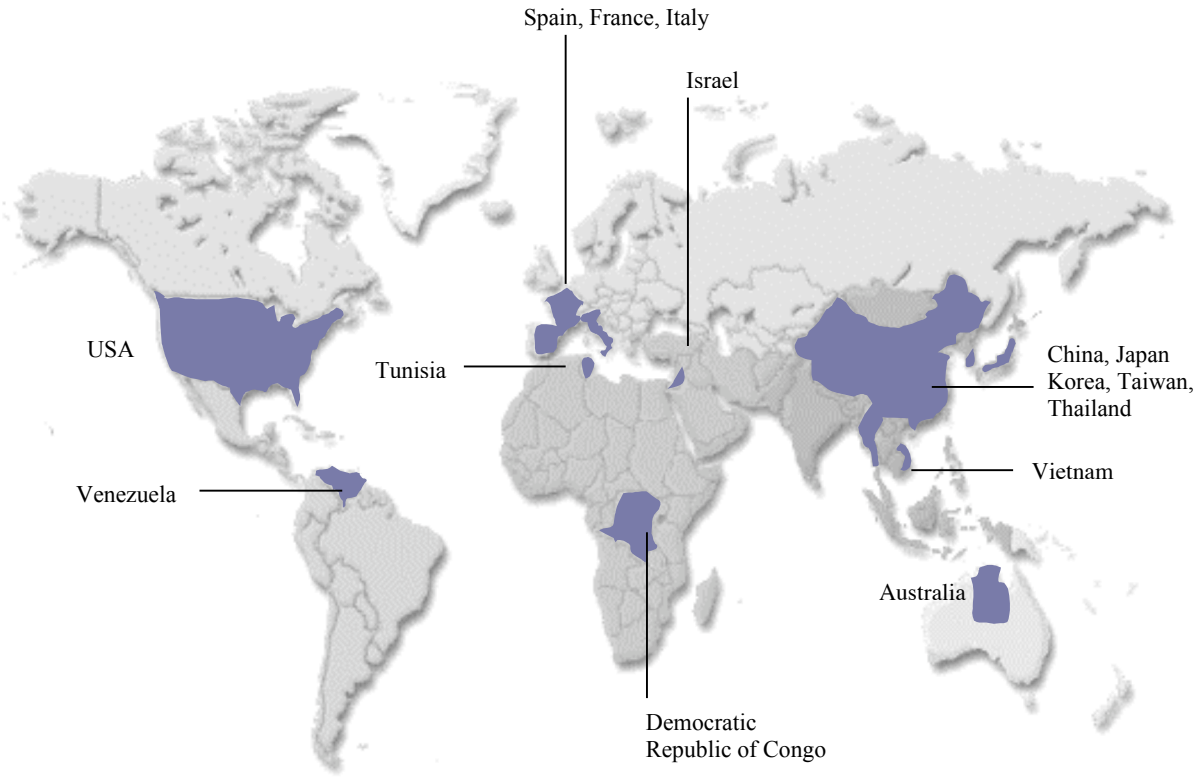


Figure 3.3

Current reported worldwide distribution of *Anaplasma platys*

Anaplasma platys is believed to be extensively distributed in the USA (French and Harvey, 1983) and has been reported in France, Italy, Greece and Taiwan (Chang *et al.,* 1996), Israel (Harrus *et al.,* 1997), Japan (Inokuma *et al.,* 2000; Inokuma *et al.*, 2001b; Inokuma *et al.*, 2001d; Motoi *et al.,* 2001; Inokuma *et al.*, 2002), China (Hua *et al.,* 2000), Spain (Sainz *et al.*, 1999), Thailand (Suksawat *et al.*, 2001a; Parola *et al.*, 2003), Vietnam (Parola *et al.*, 2003), Venezuela (Suksawat *et al.*, 2001a) and Malaysia (Irwin and Jefferies, 2004), Democratic Republic of Congo (Sanogo *et al.*, 2003) Tunisia (Sarih *et al.*, 2005). *Anaplasma*

platys has also been reported in central Australia (Brown *et al*., 2001; Brown *et al*., 2005). The full extent of its distribution in Australia has not been established.

3.7 Clinical Signs and Pathogenesis

Anaplasma platys infection is characterised by a seven to 14 day incubation after which time, clinical signs are only occasionally present (Rikihisa, 1991). It has generally been stated that dogs suffering from CICT are rarely clinically ill and do not often present with any significant haemorrhage associated with platelet depletion (Harvey *et al*., 1978; Chang *et al*., 1996; Mathew *et al*., 1997). Such a lack of clinical signs has been disputed by other studies, in which important clinical signs of weight loss, fever and depression were reported (Harrus *et al*., 1997). Harrus *et al*. (1997) provided an explanation for the differences by suggesting that there may be strains of *A. platys* in southern Europe and the Middle East that are more virulent or pathogenic than the strain present in USA. Further study is required to assess the level of virulence between strains of *A. platys*.

As suggested by the name of this disease, the most characteristic feature of *A. platys* infection is thrombocytopenia. Thrombocytopenia does not necessarily correlate to the degree of parasitemia, as it may also be attributed to immune mediation (Baker *et al*., 1987; Bradfield *et al*., 1996). An important feature of CICT is the cyclic nature of platelet infection, with thrombocytopenia occurring in cycles of approximately 10-14 day intervals (Harvey *et al*., 1978; Baker *et al*., 1987) Such repetitive absence and presence of infected platelets needs to be considered in effectively diagnosing the disease. Infection of the platelets and the resulting thrombocytopenia generally persists for seven to 10 days, followed by a time of recovery (Bradfield *et al*., 1996). CICT has also been associated with lymph node hyperplasia and plasmacytosis in various organs (Baker *et al*., 1987).

3.8 Detection and Diagnosis

3.8.1 Light microscopy

The most frequently used method of diagnosing acute *A. platys* infection is by examination of infected platelets using light microscopy (Rikihisa, 1991). Blood smears are generally stained with Giemsa and scanned for blue-staining, cytoplasmic inclusions of *A. platys* within the platelets. Notably, diagnosis using such a method is limited due to the failure of distinguishing between parasitised platelets and platelets with variable granule morphology or megakaryocyte nuclear remnants (Simpson and Gaunt, 1991). Effective diagnosis is therefore reliant on the ability and experience of the technician. In an attempt to overcome these limitations, Simpson and Gaunt (1991) optimised an immunocytochemical stain procedure for the detection of *A. platys* antigens. They developed an avidin-biotin immunoperoxidase complex immunocytochemical stain that allowed for effective discrimination between *A. platys* and other platelet inclusions using light microscopy. Detection of *A. platys* within platelets using microscopic examination of Giemsa-stained blood smears has a major limitation in that infection of platelets follows a cyclic pattern (Chang and Pan, 1996). It was suggested that a single blood smear examination may give rise to a negative diagnosis, when in fact infection exists. Multiple tests over an extended period of time may be necessary for increased accuracy.

3.8.2 Electron Microscopy

Mathew *et al.* (1997) used electron microscopy to reveal the presence of rickettsia-like inclusions within the platelets. A majority of the infected platelets showed parasitism by an organism that had a general rickettsial-like morphology. Ultrastructural studies were later conducted by Arraga-Alvarado *et al.* (2003). Due to the similarity in morphology between the *Ehrlichia* species, such a technique is limited in that it is not possible to diagnose the bacteria to a species level on the basis of morphology. Visible organisms can only be assumed to be *A. platys* due to their presence within platelets.

iii) Indirect Fluorescent Antibody Test (IFAT)

Diagnosis can also be carried out using an indirect fluorescent antibody (IFA) test. An *A. platys* IFA test was developed by French and Harvey (1983) and proved to be more effective than examination by light microscopy. This test showed some degree of specificity, as it was not able to detect antibodies to *E. canis*. Cross-reactions with antibodies of other *Ehrlichia* species have not been reported however, in the case of the *E. canis* IFAT, cross-reactivity is a significant limitation (Suksawat *et al*., 2001b; Waner *et al*., 2001).

iv) Polymerase Chain Reaction

DNA amplification using PCR has been demonstrated to show much promise as a highly sensitive and specific test for the diagnosis of *Ehrlichia* infections. Initial PCR applications were in equine and human infections (Biswas *et al*., 1991 and Anderson *et al*., 1992). Successive studies showed the successful application of PCR to the diagnosis of canine *Ehrlichia* infections. Iqbal *et al*. (1994) were first to use this technique as an effective means of diagnosing canine *Ehrlichia* infections and were able to detect *E. canis* in canine blood.

Amplification of *A. platys* DNA was first carried out by Anderson *et al*. (1992) to allow for sequencing of the DNA structure and its comparison to *E. ewingii* and other *Ehrlichia* species. This sequence information provided the initial basis for primer development in later studies. The first use of PCR to diagnose CICT was by two-step PCR in which primers were developed to target a region of the 16S rRNA gene (Chang and Pan, 1996). Sensitivity testing showed that the two-step method of PCR was 10 times more sensitive than single PCR. Chang and Pan (1996) suggest that such a PCR-based detection method could be applied to clinical use and found that PCR detected the presence of *A. platys* in blood samples that were assumed negative after Giemsa stain examination. It was also suggested that the two-step PCR was less time consuming than Southern Blot hybridisation. The PCR test was successful in detecting *A. platys* in both the acute and chronic stages of

thrombocytopenia. It has also been suggested that nested PCR (two-step) is more useful in assessing clearance of organisms after therapeutic treatment than IFAT (Wen *et al.*, 1997).

A later study developed a single step PCR for the amplification of a region of the 16S rRNA gene and was also found to be an effective diagnostic tool (Mathew *et al.,* 1997). The amplified region of DNA was sequenced to provide further support for an accurate diagnosis of *A. platys* infection. The sensitivity and specificity of this PCR was not assessed and is therefore difficult to compare with the previous two-step method. Hua *et al.* (2000) and Motoi *et al.* (2001), have more recently applied PCR to the detection of *A. platys* and allowed for the first discovery of this organism in dogs in China and Japan. One of these PCR tests was associated with the amplification of *Wolbachia* spp. (Motoi *et al.*, 2001). This suggests that the specificity of the test is limited and requires future attention. *Anaplasma platys* was also discovered in Australia, using PCR as the sole basis of detection (Brown *et al.*, 2001; Brown *et al.*, 2005). Microscopic examination failed to detect any rickettsial morulae within the platelets of the dogs and was assumed to be a consequence of the stage of the infection.

Application of reverse transcription-PCR may also have potential in *A. platys* diagnosis as this technique offers increased sensitivity than convential PCR and detects only viable organisms (Felek *et al.*, 2001). Multiplex detection of both *Ehrlichia* and *Anaplasma* spp. has been reported using Real-time reverse transcriptase PCR (Sirigireddy and Ganta, 2005)

Limited information is available on whether *A. platys* exists in a dormant phase within such organs as the spleen. If the parasites do involve other organs it may be useful to be able to detect *A. platys* in tissue samples. PCR detection of *A. platys* within tissues has not been reported, however such a technique has been used in *E. canis* diagnosis (Iqbal and Rikihisa, 1994; Harrus *et al.,* 1998).

vi) PCR detection of A. platys DNA in ticks

Inokuma *et al.* (2000) were first to successfully detect *A. platys* DNA in *R. sanguineus* ticks by using PCR. All of the ticks which they examined were semi-engorged, making it difficult to determine whether the ticks were themselves infected or whether the PCR was amplifying DNA solely within the blood-meal of the tick. Other species of *Ehrlichia* have been effectively detected in the tissues of *R. sanguineus* using PCR (Sparagano *et al.*, 1999).

3.9 Prevention and Treatment

As with all tick-borne pathogens, the most effective prevention of *A. platys* infection is avoidance of the tick vector. However, this may be difficult due to the current dispute over the actual tick species involved. The incidence of infection may be greater in the summer months due to the increase in the number of feeding ticks (Bradfield *et al.*, 1996). Therefore control measures should be more vigorous during this time. Control of vector populations by chemical treatment of dog housing and external treatment of the infected animals is suggested every 1-2 weeks in endemic areas (Rikihisa, 1991).

Administration of tetracycline hydrochloride to infected dogs resulted in the disappearance of thrombocytopenia, however some *A. platys* organisms remained in the platelets (Chang *et al.*, 1996). Oral treatment with doxycycline (5-10mg/kg for 10-14 days) was suggested to be effective in eliminating thrombocytopenia (Bradfield *et al.*, 1996). Oral treatment with doxycycline and intramuscular injection of imidocarb dipropionate resulted in a recovery 48 to 72 hours after the initiation of treatment (Harrus *et al.*, 1997). A similar co-drug treatment was administered to dogs infected with *A. platys* by Sainz *et al.* (1999) and resulted in the remission of clinical signs.

3.10 Co-infection of *Ehrlichia* and *Anaplasma* species

Ehrlichia infection can result from the simultaneous infection of dogs by multiple species. Understanding co-infection is important in avoiding incorrect diagnosis. Sainz *et al.*, (1999)

reported that dogs can be infected with both *E. canis* and *A. platys*. The infected animals were shown to present with cutaneous petechial and ecchymotic haemorrhages and treatment with doxycycline or imidocarb dipropionate resulted in remission of clinical signs. A later study demonstrated the co-infection of *E. canis, A. platys* and *E. equi* in dogs within Thailand and Venezuela (Suksawat *et al*., 2001b).

CHAPTER FOUR

General Materials and Methods

4.1 Identification of piroplasm spp. by light microscopy

Ear-tip blood, venous blood and/or buffy coat smears were stained with a modified Wright-Giemsa stain using an Ames Hema-Tek® slide stainer (Bayer AG, Germany). Smears were examined for intra-erythracytic piroplasm merozoites in the feathered region of the film or for platelet inclusions throughout the slide, initially with a low-powered objective (40x) and then at higher power (100x). At least two hundred microscopic fields of view were examined under oil immersion using the 100x objective before being reported negative (Garcia and Bruckner, 1988).

4.2 DNA extraction from canine blood

DNA was isolated from blood samples using a QIAamp® DNA mini kit (QIAGEN, Germany). 200 µl of EDTA blood was added to a 1.5 ml microcentrifuge tube containing 20 µl of QIAGEN Protease. 200 µl of AL buffer was added to the sample and mixed by pulse vortexing for 15 sec. The tube was incubated at 56 C for 10 min. Droplets formed within the lid of the tube were removed by brief centrifugation. 200 µl of 99.5% ethanol was added to the sample and mixed by pulse vortexing for 15 sec, then briefly centrifuged. The entire mixture was applied to a QIAamp spin column without wetting the rim and the closed column was spun at 6000 xg (half-speed) for 1 min. The spin column was placed in a clean collection tube and the filtrate and collection tube were discarded. 500 µl of AW1 buffer was added to the spin column, which was then centrifuged at half-speed for 1 min. After being placed in a new collection tube, 500 µl of AW2 buffer was added to the spin filter, followed

by centrifugation at 20 000 xg (full-speed) for 3 min. The filtrate was discarded and the column spun for a further 1 min. After the addition of 100 µl of AE buffer, the tube was incubated at room temperature for greater than 30 minutes to increase the DNA yield, before being centrifuged at half-speed for 1 min. Extracted DNA was frozen at -20 C.

4.3 DNA extraction from animal tissues

DNA was isolated from tissue samples using a QIAamp® DNA Mini Kit (QIAGEN, Germany). A maximum of 25 mg of tissue was macerated using a scalpel blade and placed in a sterile 1.5 ml microcentrifuge tube. Added to the tissue were 180 µl of buffer ATL and 20 µl of Proteinase K, which were then mixed by vortexing. The sample was incubated at 56 C for 4 hrs or until the tissue had completely lysed. Two hundred microliters of buffer AL was added to the tube, mixed by vortexing and then incubated at 70 C for 10 min. The tube was again centrifuged briefly and 200 µl of 100 % ethanol was added and mixed by vortexing. The entire contents of the tube was then transferred to a QIAamp spin column and the remaining protocol followed that described in section 4.3.

4.4 Gel electrophoresis

Gel electrophoresis of PCR products was performed using 1 % agarose (Promega, Madison, USA) gels in TAE buffer (40 mM Tris-HCL, 20mM acetate, 2mM EDTA). Gels were pre-stained with ethidium bromide (Amresco, USA). A 100 bp molecular weight marker (Life Technologies, Australia) was run for all gels to determine the size of PCR products. Electrophoresis was performed using a Minisub electrophoretic cell (Biorad) at 90 V for 30 min and DNA was visualised by UV transillumination.

4.5 DNA purification of gel bands

DNA purification was carried out using an UltraClean ™ Gelspin DNA Purification Kit (MO BIO Laboratories, Inc.). Amplified DNA was electrophoresed on a 1% agarose gel (90 V, 40

min). The gel was viewed under ultra-violet light and the appropriate sized band was cut out using a scalpel blade. Each band was cut out using a separate scalpel blade to avoid contamination of DNA. The bands were each placed in separate 1.5ml centrifuge tubes and the individual volume of each band was estimated. Three times the volume of the gel band of Gelbind or gel solubilization buffer was added to the gel slice and incubated for 2 minutes at 65 C. The tube was then inverted once and incubated for a further minute. The tube was inverted again to ensure mixing. This solution was then transferred to a spin filter basket and centrifuged for 10 sec at 10000 x g. The spin filter was removed from the collection tube and the eluted solution was briefly vortexed before reloading into the spin filter. The tube was centrifuged again for 10 sec at 10000xg and the flow-through liquid discarded. Three hundred microlitres of GelWash buffer was added to the filter and spun for 10 sec at 10000 x g. The flow-through liquid was discarded and the spin filter was spun for an additional 30 sec. The spin filter was carefully transferred to a clean collection tube and 50 µl of distilled water was added. After >5 min incubation at room temperature, the tube was centrifuged for 30 sec at 10000 x g. Eluted DNA was immediately used for sequencing amplification or was frozen at –20 C for later use.

4.6 Sequencing amplification

DNA was sequenced using an ABI Prism® Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California) according to manufacturer's instructions, with the following modifications: Amplification was carried out in a 10 µl reaction mixture containing the following: 0.5 µl each of the forward and reverse primers diluted to 3.25 pmol/µl, 2.0 µl of dye terminator solution, 2.0 µl of half term (Genpak Inc., Stony Brook, New York) and 5.5µl of purified template (from 3.4). Forty cycles of amplification (94 C for 10 sec, 60 C for 5 sec and 60 C for 4min) was preceded by an intial denaturation of 94 C for 2 min 20 sec and followed by a holding temperature of 15 C.

4.7 Purification of sequencing reactions

Twenty-five microlitres of 95% ethanol, 1 µl of 125 mM EDTA and 1 µl of 3M sodium acetate were added to a 0.6 ml tube. The 10 µl amplified DNA was centrifuged briefly and added to the ethanol mixture. This solution was mixed gently using a pipette, then incubated on ice for 20 min and then centrifuged for 30 min. The supernatant was carefully removed with a pipette and 125 µl of 80 % ethanol was added to the remaining precipitate. The solution was gently 'rolled' to ensure all salt deposits were removed from the sides of the tube. The tube was spun for 5 min at 20 000 xg. The majority of the ethanol was removed with a pipette and the sample was vacuumed dry in a vacuum desiccator (Nalgene).

4.8 Analysis of sequence chromatograms

The sequenced products were analysed using the program SeqEd v.1.0.3 (ABI) and were compared to sequence data available from GenBank®, using the BLAST 2.1 program (http://www.ncbi.nlm.nih.gov/BLAST/).

4.9 Immunofluorescent Antibody Test (IFAT)

IFAT was performed using a modified procedure described by Anderson *et al*., (1980). The antigen used was a pre-prepared, ethanol fixed glass slides coated with a thin layer of *B. gibsoni*-infected erythrocytes (approximately 6% parasitaemia) obtained from *in vivo* culture of parasite from a naturally infected pit bull terrier in Victoria, Eastern Australia. Slides were stored at -70 C until required. The conjugate used was Rabbit anti-canine IgG globulin labelled with FITC is diluted in PBS at 1:1000.

Antigen slides were thawed at room temperature. Patient serum was diluted 1: 40, 1: 160, 1; 640, 1: 2560 and 1: 10240 and then placed in demarcated areas of the slide and incubated at 37 C for 60 min, then washed with PBS and rinsed. Diluted conjugate was applied to each sample, incubated at 37 C for 60 min, washed in PBS for 10min, dried, then overlaid with

buffered glycine. Each slide was then cover slipped and examined using a fluorescent microscope. Positive (serum from a known-infected dog supplied by Onderstepoort Veterinary Institute, South Africa) and negative controls (SPF-canine serum) were used on each slide during each test. Samples were considered positive with a titre greater than 1:40.

CHAPTER FIVE

Development of a PCR-RFLP assay for the detection and differentiation of the canine Piroplasmida species and evaluation of Whatman® FTA cards

5.1 Introduction

Accurate detection is imperative to the identification of the species of pathogen responsible for infection. Many reports suggest PCR is a highly effective detection technique and could potentially be used in the routine diagnosis of diseases such as piroplasmosis (McLaughlin *et al*., 1992; Prichard and Tait, 2001). It is important however to consider the low potential of PCR in regions of the world where economic resources and sophisticated technology are limited (Hanscheid and Grobusch, 2002). The application of blood samples to FTA cards may overcome this limitation, by allowing samples to be sent rapidly and safely to diagnostic facilities capable of PCR-based diagnosis.

Multiple species of piroplasm are now recognised to infect dogs, including three subspecies of *B. canis, B. gibsoni, B. conradae, T. annae*, an unnamed *Babesia* sp. from North Carolina and possibly *T. equi*. The morphological similarity between these species and subspecies of the canine piroplasm has led to much confusion over accurate diagnosis using light microscopy (Kjemtrup *et al*., 2000a). Likewise, there are reports that serology-based diagnosis also lacks specificity, with the existence of antigen cross-reactivity between species and even between genera with methods such as immunofluorescent antibody tests (IFAT) (Yamane *et al*., 1993). Various PCR-based tests have been developed for detection

of the canine piroplasms (Zahler *et al.*, 1998; Carret *et al.*, 1999; Ano *et al*, 2001; Birkenheuer *et al.*, 2003a) however, many are species-specific and fail to detect novel species and genotypes of canine piroplasm. Some tests require complete sequencing of the amplified target gene to determine the species or genotype present. Most assays have targeted the 18S rRNA gene; a conserved, functional gene that contains moderate levels of genetic variation that can be used to discriminate between species. This gene is also found in multiple copies within the genome, allowing for increased levels of detection when amplified using PCR (Hillis and Dixon, 1991). Restriction fragment length polymorphism (RFLP) analysis offers an effective means of discriminating between species without the need for DNA sequencing. To date, no PCR-RFLP for the differentiation of all reported canine piroplasm species has been developed.

Blood samples are often difficult to store and transport from remote locations or places with limited technology resources (Zhong *et al.*, 2001). It is therefore beneficial to use a method that requires minimal expertise and equipment, while also being simple and cost effective to transport samples worldwide. In addition, long-term storage of samples such as whole blood can be difficult due to the space and freezer requirements. Repetitive freeze thawing of blood samples can also result in degradation of DNA, decreasing the sensitivity of DNA amplification (Farnert *et al.*, 1999). The application of samples to FTA cards may help to overcome these problems. The FTA matrix also contains a substance that allows for the inactivation of pathogens such as bacteria and viruses (Moscoso *et al*, 2004). This enables FTA samples to be sent domestically and internationally without the risk of spreading disease pathogens and also minimizing the risk of infection to laboratory personnel. Multiple studies have demonstrated the antimicrobial efficacy of the FTA treated matrix (Moscoso *et al.*, 2004; Li *et al.*, 2004). Currently, Australian quarantine laws prohibit the importation of whole canine blood from countries including unless UV sterilized (http://www.aqis.gov.au/, accessed 5/2002), inactivating potential pathogens but also cross-links DNA. FTA cards

offer a method of importing blood samples without the need for UV sterilization and also minimizes the risk of importing infectious pathogens.

This chapter describes the development of a PCR-RFLP assay for the detection and differentiation of the canine piroplasm species. It also describes the assessment of FTA cards for the application of canine blood samples for subsequent PCR amplification of piroplasm DNA, thereby allowing for infected blood samples to be imported into Australia from overseas countries for molecular characterisation (refer to Chapter ten). The Whatman® FTA DNA purification method was also compared to QIAGEN DNA extraction of blood applied to filter paper and IsoCode® Stix DNA isolation techniques.

5.2 Aims

i. To design a PCR-RFLP assay for the detection and differentiation of the canine piroplasm spp.

ii. To determine the detection limit and specificity of the PCR-RFLP assay

iii. To assess whether piroplasm DNA could be amplified from blood applied to FTA cards

iv. To determine the possibility of DNA cross-over contamination using the Whatman® FTA template preparation protocol

v. To compare the detection limit of FTA disc purification, QIAGEN extraction and Isocode® Stix template preparation

5.3 Materials and Methods

5.3.1 Primer design

A nested set of primers was designed to amplify a partial region of the 18S rRNA gene of both *Babesia* and *Theileria* species. A Clustal w (Thompson *et al*., 1994) alignment was performed using complete 18S rRNA gene sequences of *Babesia gibsoni* (AY278443), *Babesia conradae* (AF158702), *Theileria annae* (AF188001), *Babesia canis vogeli* (AB083374), *Babesia canis canis* (AY072926), *Babesia canis rossi* (L19079), *Babesia* sp. (North Carolina) (AY618928), *Theileria equi* (AY150064), *Babesia felis* (AF244912), *Babesia microti* (AB070506), available from the GenBank database. An external and an internal set of primers (Table 5.1) were designed on the basis of conserved regions of DNA between the aligned sequences using Amplify 2.1 (Engels, W., University of Wisconsin, Madison). The external primer set amplified an approximately 930 bp product, while the internal set amplified an approximately 800 bp product.

Primer name	Sequence
BTF1 (external)	5’ GGCTCATTACAACAGTTATAG 3’
BTR1(external)	5’ GAGAGAAATCAAAGTCTTTGGG 3’
BTF2 (internal)	5’ CCGTGCTAATTGTAGGGCTAATAC 3’
BTR2 (internal)	5’ CGATCAGATACCGTCGTAGTCC 3’

Table 5.1

External and Internal primer sets for the amplification of a partial region of the 18S rRNA gene of most Piroplasmida species

5.3.2 Restriction fragment length polymorphism design

A restriction fragment length polymorphism (RFLP) technique was designed to permit discrimination between each of the canine piroplasm species and, in particular, *Babesia*

canis and *Babesia gibsoni* in blood samples from dogs in Australia. Complete sequences of the 18S rRNA gene each of the canine species and subspecies available on GenBank database were imported into the program Amplify 2.1 (Engels, W., University of Wisconsin, Madison) and the target region of DNA was determined using the internal primer set (BTF2 and BTR2). The sequence of the amplified internal PCR product was then used in DNA Strider™ 1.0 (Mark, 1988) to determine the most suitable restriction enzymes for discriminating between the canine piroplasm species.

5.3.3 DNA extraction and amplification

DNA was isolated from 200 µl aliquots of EDTA blood (stored at -20 C) using a QIAamp® DNA mini kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions (refer to Chapter four, section 4.2). One µl of extracted DNA was added to a 24 µl reaction mixture comprising 0.6875 units of Tth Plus DNA polymerase (Fisher Biotech, Australia), 200 µM of each dNTP, 12.5 pmoles of the forward and reverse primers (Invitrogen, Australia), 2.5 µl 10x PCR buffer (Fisher Biotech, Australia) and 1.5 µl $MgCl_2$ (Fisher Biotech, Australia). Positive (1 µl of *B. canis vogeli* DNA, Australia) and negative (1 µl dH_2O) control samples were included with each set of PCR reactions.

Amplification was performed on a GeneAmp PCR system 2700 thermal cycler (Applied Biosystems, USA). For the primary round of amplification, an initial activation step at 94 C for 3 min, 58 C for 1 min and 72 C for 2 min, was followed by 45 cycles of amplification (94 C for 30 sec, 58 C for 20 sec and 72 C for 30 sec) and a final extension step of 72 C for 7 min for 25μl reactions. The same conditions were followed for the secondary round of amplification, except that the annealing temperature was increased to 62 C, using 1μl of DNA template from the primary reaction. Amplified DNA was electrophoresed and visualised according to the method described in Chapter four, section 4.4.

5.3.4 Restriction digestion

Six µl of amplified DNA from the secondary PCR reaction was subjected to restriction enzyme digestion in a reaction mixture of 16.3 µl of dH_2O, 2.0 µl of Buffer B (Promega, Madison, USA), 0.2 µl of Bovine serum albumin acetylated (Promega, USA). The reaction mixture was gently mixed and 0.5 µl of the appropriate restriction enzyme (either Hinf I, Hinc II or Ava II) (Promega, USA) was added and then incubated at 37 C for 2 hrs. Restriction products were then electrophoresed at 80 volts for 1 hr on a 3 % agarose gel (Promega, USA) stained with ethidium bromide and visualised using UV illumination.

5.3.5 DNA Sequencing

Amplified products were purified using an UltraClean™ Gelspin DNA Purification Kit (MO Bio Laboratories, Inc., Sohlana Beach, California) and sequenced using an ABI Prism™ Dye Terminator Cycle Sequencing Kit (Applied Biosystems [ABI], Foster City, California) (Refer to Chapter four, section 4.6). The sequenced products were analysed using SeqEd v.1.0.3 (ABI), compared with sequence data available from GenBank™ using the BLAST 2.1 program (http://www.ncbi.nlm.nih.gov/BLAST/), and aligned to sequences available from GenBank™ using Clustal w (Thompson *et al*., 1994).

5.3.6 Determination of detection limit of PCR

a) Serial dilution of infected blood

The sensitivity of the PCR was calculated by performing PCR assays on a blood sample with a known parasitaemia that had been serially diluted in parasite free canine blood as previously described (Jefferies *et al*., 2003). A venous blood sample from a dog known to be infected with *Babesia* by microscopic examination was collected and EDTA was added. A thin blood smear was prepared from venous blood and the parasitaemia was calculated according to Read and Hyde (1993), by counting between 500 to 1000 erythrocytes and noting the number that were infected with *Babesia* (cells infected with more than one

piroplasm were counted as one). A total of 1187 erythrocytes were counted over a thin blood smear section of uniform density.

The blood sample of known parasitaemia was then diluted using canine blood considered to be free from *Babesia* infection by microscopy, PCR and the dog's lack of exposure to tick vectors. Twenty-five microlitres of infected blood was added to 225 µl of uninfected control blood and mixed. 25 µl of this blood was then added to 225 µl of control blood to form a $1x10^{-2}$ dilution. This procedure of serial dilution was repeated until a $1x10^{-7}$ dilution was obtained. DNA was extracted from 200 µl of each of the diluted blood samples according to the described extraction procedure in Chapter four, section 4.2. Amplification of the extracted DNA was carried out according to the procedure described in section 5.3.3.

b) Serial dilution of quantified plasmid DNA

i) Cloning of primary PCR product

The primary PCR product amplified from an isolate of *B. canis vogeli* was cloned into a plasmid vector using a TOPO® Cloning Kit (Invitrogen, California). TOPO® cloning reactions were comprised of 4 µl of purified PCR product, 1 µl of salt solution (1.2M NaCl and 0.06M $MgCl_2$) and 1 µl of TOPO® vector. The reactions were mixed gently and incubated at room temperature for 30 min before being placed on ice for 2 min.

Transformation involved the addition of 2 µl of the TOPO® cloning reaction to 1 µl of OneShot® chemically competent *E. coli* cells, mixed gently, incubated on ice for 30 min and then heat shocked at 42 C for 30 sec. Cells were again incubated on ice, before 200 µl of SOC media (at room temperature) was added and incubated at 37 C for 1 hr with continual shaking. A 100 µl aliquot of the transformation mixture was then spread evenly onto LB agar plates containing ampicillin (50 µg/ml) and then incubated at 37 C overnight. Ten colonies were chosen to be screened for the insert, with half of each colony being screened

by PCR, while the other half was inoculated into 200 µl of LB media containing 50 µg/ml of ampicillin and incubated at 37 C overnight with shaking.

ii) PCR screening of colonies

The primers M13 Forward (5' GTAAAACGACGGCCAG 3') and M13 Reverse (5' CAGGAAACAGCTATGAC 3') were used to amplify positive transformants. Samples from each of the 10 colonies selected were added to a 48 µl PCR reaction mixture. After an initial denaturation step at 95 C for 5 min, 35 cycles of amplification were conducted (95 C for 20 sec, 56 C for 20 sec, 72 for 1 min) before a final extension step of 72 C for 7 min. Transformants were sequenced according to the method described in Chapter four, section 4.6.

iii) Plasmid extraction

Samples determined to be positive for the desired insert were purified using a QIAprep® Plasmid Miniprep Kit (QIAGEN, Germany). Cells inoculated into LB media were pelleted by centrifugation at low speed (1450 xg) for 1 min. Pelleted cells were transferred into a sterile microcentrifuge tube and resuspended in 250 µl of Buffer P1. Two hundred and fifty µl of Buffer P2 and then 350 µl of Buffer N3 were added to the sample, gently mixed four to six times on addition of each buffer. Samples were then centrifuged for 10 min at high speed (20 000 xg) and the resulting supernatant was transferred into a QIAprep® spin column, centrifuged at high speed (20 000 xg) for 1 min and the flow through liquid was discarded. The spin column was washed with 750 µl of Buffer PE and then centrifuged at high speed for 1 min. Flow through was discarded and the spin column was centrifuged for a further 1 min. The QIAprep® column was placed into a clean 1.5 ml microcentrifuge tube and 50 µl of Buffer EB was added to the column, before being left to incubate at room temperature for 1min. The sample was then centrifuged at high speed for 1 min and the flow through retained for subsequent analysis.

iv) Quantification and dilution of plasmid DNA

The transformed plasmid DNA was quantified using a Lambda 25 UV/VIS spectrometer (PerkinElmer). The purified sample contained 0.035 ug/ml of DNA and was serially diluted 10 fold to a dilution of $1x10^{-10}$. The diluted samples were amplified using the nested PCR.

5.3.6 Determination of specificity of PCR-RFLP

The specificity of the primers used was determined by BLAST searching the primer sequences to make sure that they did not amplify host or human DNA or other blood microbe DNA. The primers of this PCR assay were designed to amplify most species of the genera *Babesia* and *Theileria* based on 18S rRNA sequences available on the GenBank database (http://www.ncbi.nlm.nih.gov/entrez/, accessed 12/2002). Speciation is then achieved using RFLP. In addition, a sample containing both *B. canis vogeli* and *B. gibsoni* DNA was subjected to PCR-RFLP to assess the detection of co-infections of multiple *Babesia* species. Sixteen blood samples obtained from dogs in New Zealand, a country considered free of all canine piroplasm species, were also screened using the PCR-RFLP as an additional negative control.

The specificity of the PCR-RFLP assay was also determined by amplifying DNA from various piroplasm species and DNA obtained from cultured *Neospora caninum* and *Toxoplasma gondii* and other parasite species (Table 5.2).

Species name	Geographical origin	Contributor
Babesia canis vogeli	Australia	This study
Babesia canis canis	France	Peter Irwin, Murdoch University
Babesia canis rossi	South Africa	Linda Jacobson, University of Pretoria, South Africa
Babesia gibsoni	Australia	This study
Babesia gibsoni	North Carolina	Ed Breitschwerdt, NCSU, USA
Theileria annae	Spain	Angel Criado-Fornelio, Universidad de Alcala, Alcala de Henares, Spain
Babesia microti	Unknown	Louise Jackson, Tick Fever Research Centre, Qld, Australia
Neospora caninum	Australia	Linda McInnes, Murdoch University
Toxoplasma gondii	Australia	Linda McInnes, Murdoch University
Plasmodium falciparum	Unknown	Chee Kin Low, Murdoch University
Dirofilaria immitis	Australia	Russ Hobbs, Murdoch University

Table 5.2

Details of protozoan and other specificity control DNA used to test the specificity of the PCR-RFLP assay.

5.3.7 Evaluation of FTA

i) Blood samples

A *B. canis vogeli* infected blood sample with a known parasitaemia (27 %) was serially diluted into non-infected blood (refer to section 5.3.6).

ii) Application of canine blood to FTA® Classic Cards

FTA® Classic Cards (Whatman International Ltd, UK) were cut into one cm wide strips (vertically) using a sterile blade to avoid DNA contamination of FTA paper (Figure 5.1). This enabled more efficient use of the FTA Classic Cards, increasing the number of samples used per card and minimizing cost. EDTA blood was applied to the FTA cards according to the manufacturers instructions (Whatman International Ltd, Kent, UK) and allowed to air dry. Samples were then stored at room temperature in a sealed plastic bag containing a silica

desiccant, until subsequently analysed. A 1.2 mm Harris Micro Punch was used to cut discs from the FTA cards and transfer to a PCR tube for later processing.

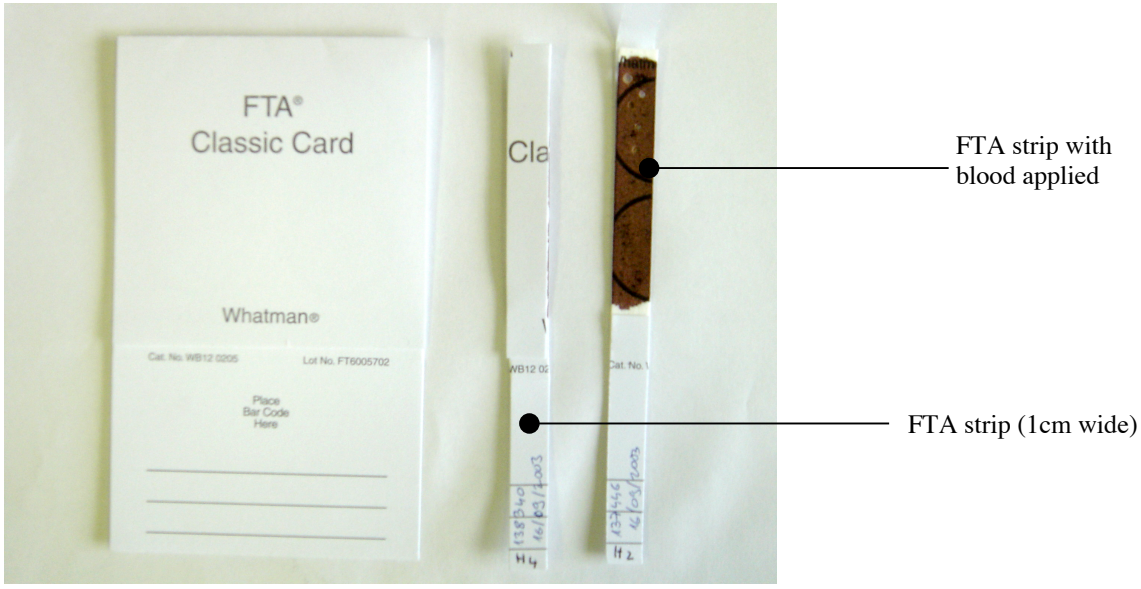


Figure 5.1

Example of blood samples applied to a FTA® classic card (cut into strips)

iii) Assessment of cross-over contamination risk

In order to ensure that there is no cross-contamination between samples, the Whatman® FTA protocol recommends rinsing the tip of the punch with ethanol between samples and drying with a sterile wipe or taking a single punch from sterile blank filter paper between samples. A modified cleaning protocol combining both suggested methods was assessed by conducting the following procedure in triplicate. For each assessment, a disc was punched from a FTA card applied with known *Babesia* positive blood. The punch was then rinsed with 70% ethanol and dried with a sterile wipe. Six discs were then punched from a sterile sheet of filter paper, with each disc being placed in a separate 0.2 ml tube for subsequent PCR amplification.

iv) Whatman® preparation technique of DNA for PCR analysis

For each sample, a 1.2 mm disc was placed into a 0.2 ml tube, 200 µl of FTA Purification Reagent was then added to the tube and incubated at room temperature for 10 min. All spent FTA Purification Reagent was then removed and discarded using a pipette. A further two washes of the disc using FTA Purification reagent was carried out. 200 µl of TE Buffer was added to the sample disc and incubated at room temperature for 10 min, before being removed using a pipette and discarded. A second wash using TE was then performed. The disc was then dried using a vaccum dessicator (Nalgene) for 30 min before performing PCR.

v) Comparison of FTA with IsoCode™ Stix and QIAGEN extraction methods

Sensitivity of each method was calculated by serially diluting canine blood with a known percentage *Babesia* parasitemia (according to section 5.3.6). Blood of each dilution was then applied to separate FTA strips or IsoCode™ Stix and a PCR was conducted from samples prepared by each of the following two methods.

a) QIAamp DNA isolation protocol

Serially diluted blood was applied to For each sample a 2 cm strip of blood covered FTA paper was placed in a centrifuge tube with 40 µl of proteinase K and 180 µl ATL buffer, then incubated at 56 C for 1 hr. 200 µl of AL buffer was then added to the sample, mixed by pulse-vortexing for 10 sec and incubated at 70 C for 1 hr. The tube was briefly centrifuged, then all of the lysate was carefully removed and applied onto a QIAamp minelute column and centrifuged at 6000 xg for 1min.

b) IsoCode™ Stix preparation

Approximately 10 µl of *Babesia* infected blood (serially diluted) was applied to the IsoCode Stix (Schleicher and Scheull, Germany) and dried at room temperature for 3 hrs. Each blood-covered triangle was placed over an open sterile microcentrifuge tube and detached while

closing the lid, allowing the triangle to fall to the base of the tube. Samples were then washed with 500 μl of dH_2O by pulse vortexing three times for a total of five sec. Sterile fine point forceps were then used to remove the matrix from the wash and gently squeezed against the side of the tube to remove excess liquid. The matrix was then transferred to a new 0.5ml tube, immersed with 50 μl of dH_2O and heated at 95 – 100 C for 15 – 30 min. The sample was pulse vortexed 60 times, then briefly centrifuged and the matrix removed, squeezing to remove excess liquid. Five μl of the remaining eluate was used as the PCR template DNA.

vi) PCR amplification

Piroplasmida sp. DNA was amplified using the nested-PCR assay described in section 5.3.3.

5.4 Results

5.4.1 Determination of detection limit of PCR

The PCR assay was estimated to detect a parasitemia of 2.7 x 10^{-6} % for the primary round of amplification and 2.7 x10^{-7} % in the secondary round of amplification using serially diluted blood (Figure 5.2).

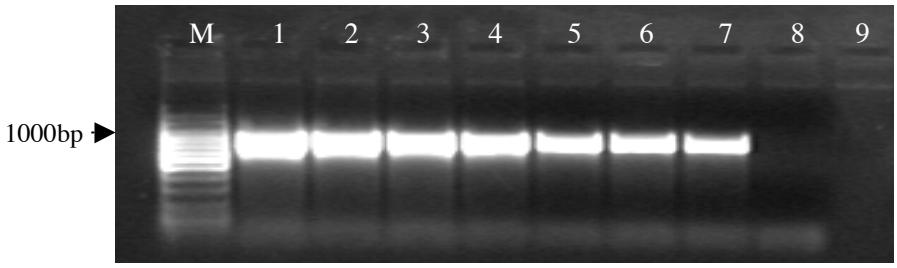


Figure 5.2

Sensitivity of PCR assay (secondary round) using serial dilution of canine blood. M - Molecular marker, 1 - Neat dilution of *Babesia* infected blood, 2 to 8 - serial dilutions of A, 1x10^{-1}- 1x10^{-7}, 9 - negative control.

The detection limit using serial dilution of cloned primary PCR product was calculated to be 12 molecules of DNA for the primary PCR and 1.2 molecules for the secondary round of amplification (Figure 5.3).

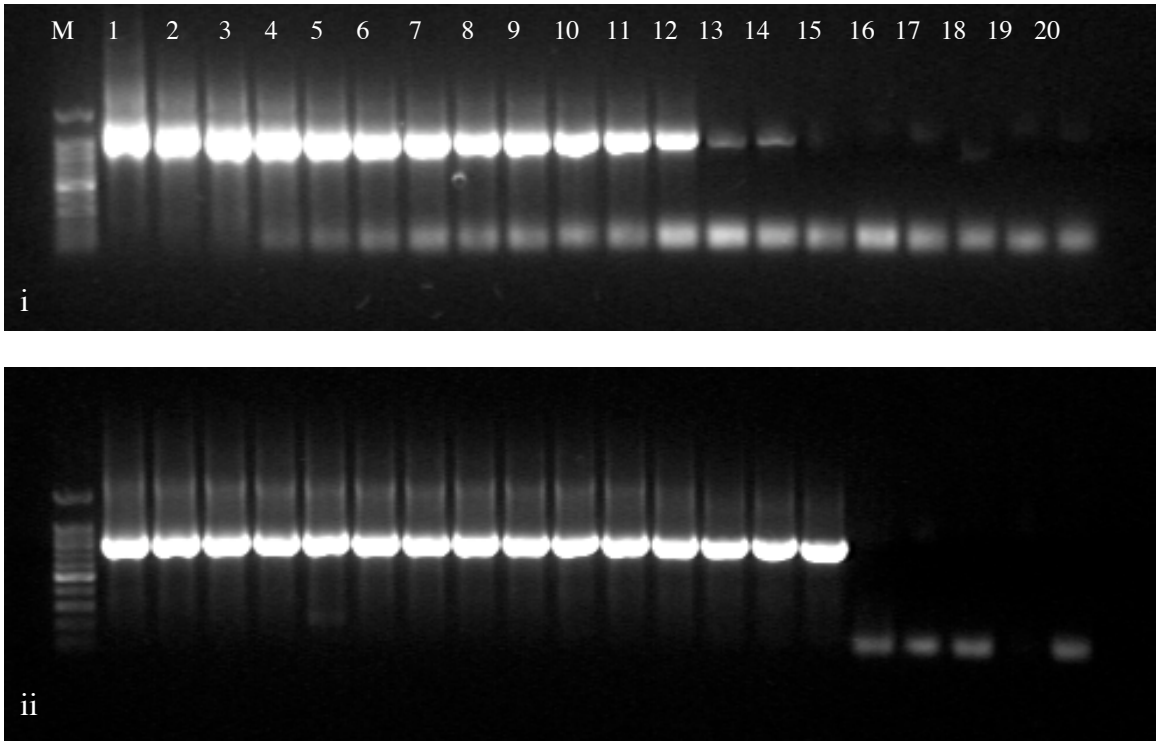


Figure 5.3

Detection limit of primary round PCR (i) and secondary round PCR (ii) using cloned product (M – molecular marker, 1 to 20 – serially diluted samples, neat to10^{-18}).

5.4.2 Specificity of PCR-RFLP

Using a three-stage screening system, each of the canine piroplasm species can effectively be discriminated with the designed PCR-RFLP assay. Amplification using the secondary (internal) set of primers allows for the separation of *T. annae, T. equi* and *B. conradae* from the remaining canine species on the basis of the larger PCR product produced (Table 5.3). Further distinction can be established through the restriction digestion of the secondary PCR product with Hinf (Table 5.3). The banding patterns for only *B. canis canis, B. canis rossi,*

B. canis vogeli and *B. gibsoni* are shown in Figure 5.4, with the remaining species being unavailable for testing. The PCR-RFLP assay was also able to detect more than one species within a single sample as shown by the detection of both *B. gibsoni* and *B. canis* (Figure 5.4).

Piroplasm spp.	**2º PCR (bp)**	**No of RFLP products**	**Product sizes (bp)**
Babesia canis vogeli	794	4	592, 102, 80, 18
Babesia canis canis	795	4	593, 102, 80, 18
Babesia sp. (North Carolina)	784	4	584, 102, 78, 18
Babesia canis rossi	795	5	303, 289, 102, 81, 18
Babesia gibsoni	794	5	321, 270, 102, 81, 18
Theileria equi	833	5	329, 283, 111, 90, 18
Theileria annae	849	6	486, 139, 111, 59, 34, 18
Babesia conradae	843	6	483, 139, 112, 56, 34, 17

Table 5.3

Expected RFLP product sizes using the restriction enzyme Hinf I for each of the canine piroplasm species.

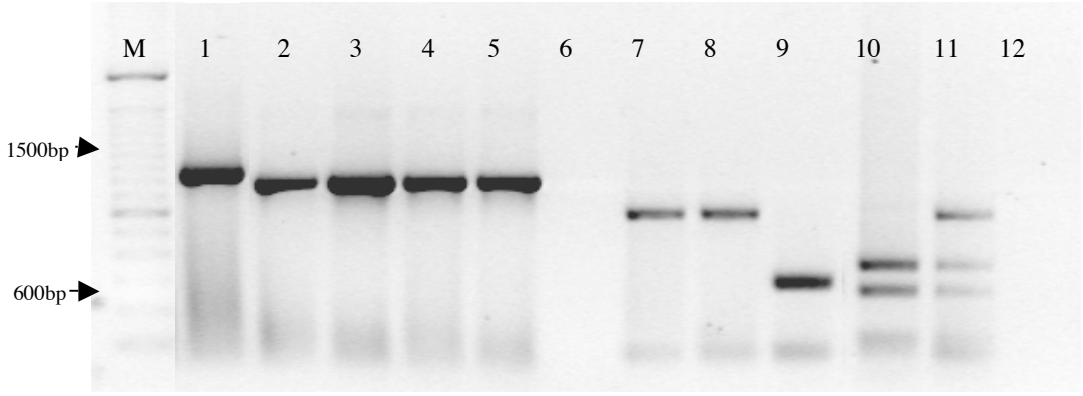


Figure 5.4

RFLP banding patterns for selected canine piroplasm species (inverted colour display). M – molecular marker, lanes 1-5 – Secondary PCR product, lanes 7-11 – RFLP products (1 – *Theileria annae*, 2/7 –*B. canis canis*, 3/8 – *B. canis vogeli*, 4/9 – *B. canis rossi* , 5/10 – *B. gibsoni* , 11 – *B. canis vogeli* and *B. gibsoni*, 12 – negative control).

Differentiation between *B. canis canis* and *B. canis vogeli* was produced using the restriction enzyme HinC II (Table 5.4), while *T. annae, T equi* and *B. conradae* were separated using Ava II (Table 5.4).

Restriction enzyme	Species	No of products	Product sizes
HinC II	*Babesia canis vogeli*	1	794
	Babesia canis canis	2	463, 330
Ava II	*Babesia conradae*	3	636, 173, 32
	Theileria annae	1	849

Table 5.4

Discrimination of the canine piroplasm species using the restriction enzymes HinC II and Ava II.

The PCR assay was also found to amplify piroplasm species beyond just the canine species as observed by the amplification of *Babesia microti* DNA. The secondary product amplified from *B. microti* was of similar size to *T. annae* (851bp). *Neospora caninum* and *T. gondii* was also amplified by the PCR assay, however the secondary product was larger (871bp) than that amplified for each of the canine piroplasms (which ranged from 784 to 849bp). The PCR assay did not amplify DNA from the other tested protozoan species, *Dirofilaria immitis* or the host (*Canis familiaris*).

5.4.3 Evaluation of FTA

i) Assessment of punch cross-over contimination

Each of the trials used to assess punch cross-over contamination amplified DNA from the negative punch discs. The greatest number of blank disc punches taken before no DNA was amplified was 2 for two trials and 3 within the third trial (Figure 5.5). Six negative control punches using sterile filter paper were taken between each sample and PCR was carried out on the sixth sample as an added negative control for all samples that were subsequently assessed.

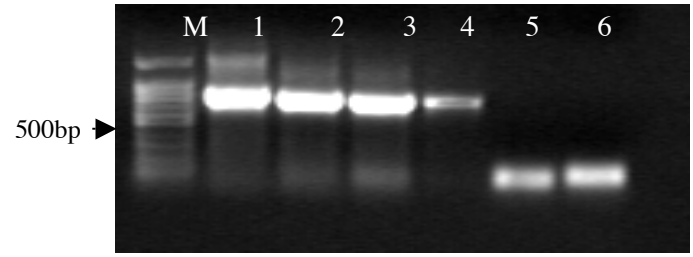


Figure 5.5

Secondary PCR assessment of disc punch cross-over contamination risk (M – molecular marker, 1- *B. canis* positive blood sample, 2-6 - negative discs punched subsequent to positive sample)

ii) Detection limit of FTA-PCR technique

The limit of detection using the Whatman method of purification of the FTA discs and PCR amplification was determined to be equivalent to a blood sample with a 2.7 x 10^{-4} percentage parasitemia for the primary PCR and 2.7 x 10^{-5} for the secondary assay (Figure 5.6)

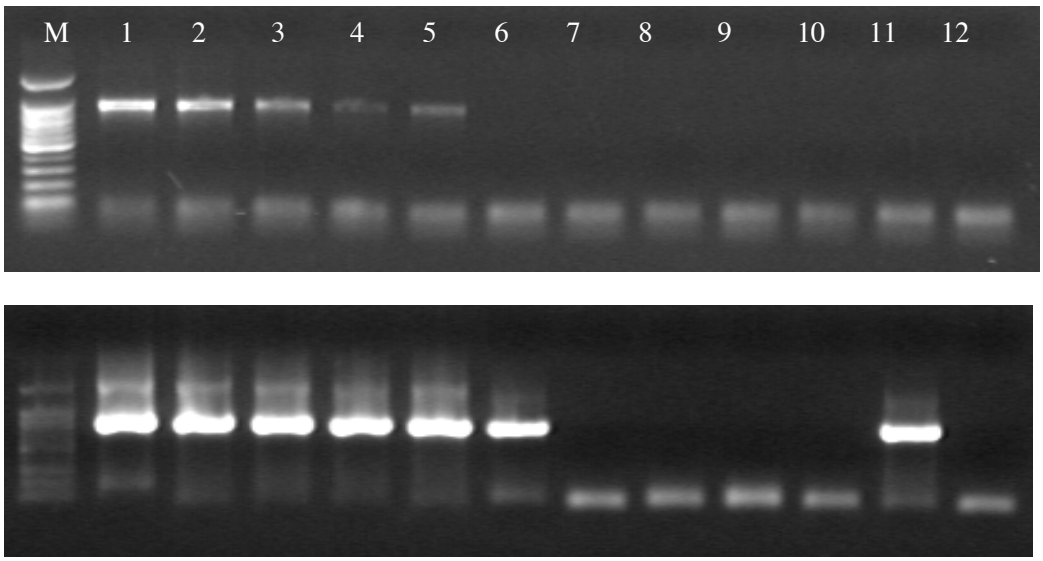


Figure 5.6

Detection limit of FTA discs (Whatman method) – (i) Primary PCR and (i) Secondary PCR (M – molecular marker,1 – neat blood sample, 2-10 serial dilution of blood 1x10^{-1} – 1x10^{-9}, 11 – positive control, 12 – negative control).

9.3.5 Detection limit of QIAGEN and IsoCode Stix DNA preparation techniques

The QIAGEN extraction technique of the FTA blood samples produced a detection limit of 2.7 x 10^{-3} and 2.7 x10^{-4} % parasitaemia for the primary and seconday PCR respectively (Figure 5.7). The highest level of detection for the Isocode Stix method was a 2.7 x10^{-5} % parasitaemia for the secondary PCR (Figure 5.8).

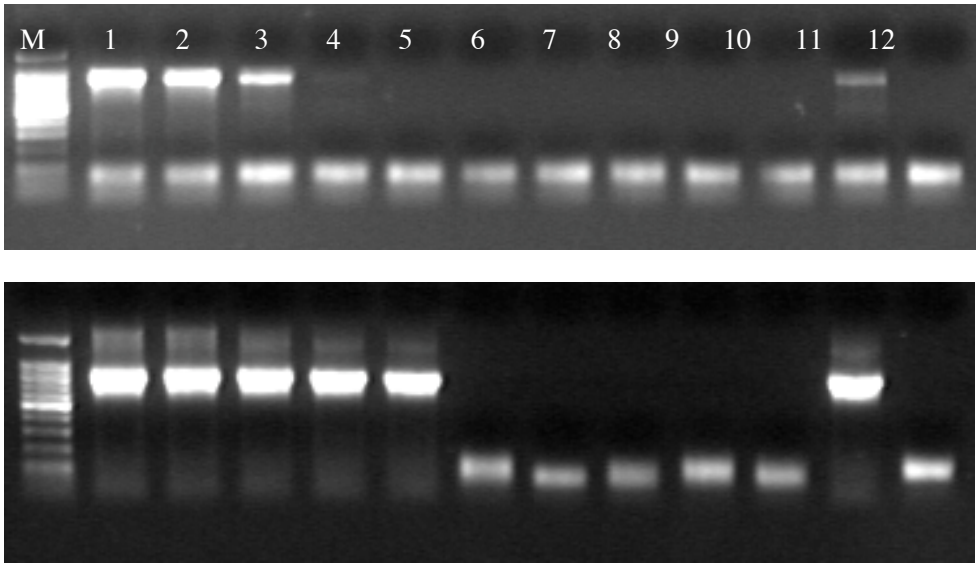


Figure 5.7

Detection limit of FTA strips using QIAamp extraction technique – (i) Primary PCR and (ii) Secondary PCR (M – molecular marker, 1- neat blood sample, 2-10– serial dilution of blood 1x10^{-1} – 1x$10^{-9,}$, 11 – positive control, 12 – negative control)

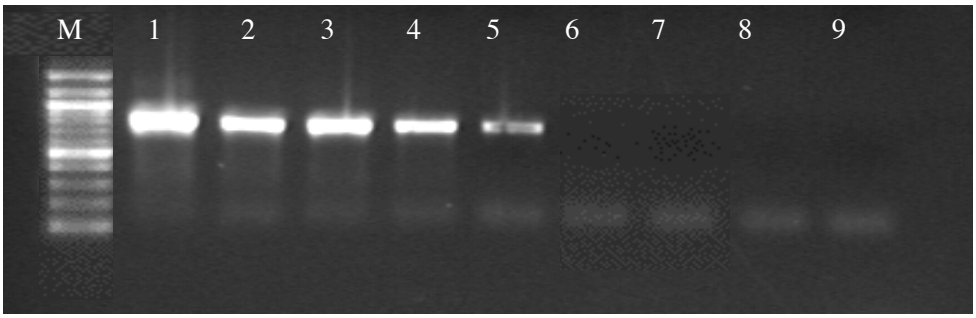


Figure 5.8

Detection limit of the secondary PCR using serially diluted blood on Isocode Stix (M – molecular marker, 1- neat blood sample, 2-9 – serial dilution of blood $1x10^{-1}$ – $1x10^{-8}$)

5.5 Discussion

5.5.1 PCR-RFLP for the detection and differentiation of the canine piroplasm spp.

Although RFLP-based assays for the detection of canine *Babesia* have previously been published, each was designed for the differentiation of the *B. canis* subspecies (Citard *et al*., 1995; Zahler *et al*., 1998; Carret *et al*., 1999) or *B. gibsoni* and *B. conradae* (Macintire *et al*., 2002). In this study a technique has been developed that is capable of detecting and discriminating all of the reported canine piroplasm species without the need for sequencing and also allows for the detection of co-infections by more than one species of piroplasm. Such an assay should be considered superior to PCR-based methods designed to detect a single species in situations including the routine screening of samples in a veterinary diagnostic laboratory or for quarantine and biosecurity measures. The PCR developed in this study can potentially amplify all members of the order Piroplasmida and also closely related apicomplexan species, enabling novel species and/or genotypes of canine piroplasms that may not yet have been genetically characterised to be amplified. Ambiguous RFLP results can then lead to the amplified product being sequenced and correct speciation can be determined.

The moderate level of genetic variation found within the 18S rRNA gene of the canine piroplasm species allows for effective discrimination between species using the RFLP technique. While this PCR-RFLP assay is beneficial in discriminating easily between certain species, such as *B. gibsoni* and *B. canis vogeli* through the use of a single restriction digest, differentiating between all canine species using multiple restriction digestions is somewhat laborious. A less labour intensive method could be acheived by the modification of the described PCR assay into a quantitative method, such as quantitative real-time PCR based on Taqman or SYBR Green chemistries (Giglio *et al*., 2003; Jeong *et al*., 2003) and warrants further study. Loop-mediated isothermal amplification has also been reported as a rapid and sensitive detection tool (Ikadai *et al*., 2004) however was reported to be species-specific and additional research is necessary to determine whether this technique could be used to detect multiple species including possible novel species and genotypes.

PCR is considered to be one of the most sensitive diagnostic methods currently available for the detection of species of canine *Babesia* (Ano *et al*., 2003; Birkenheuer *et al*., 2003; Jefferies *et al*., 2003; Inokuma *et al*., 2004). The detection limit of the assay reported in this chapter, as a value of percentage parasitaemia, is higher than PCR assays previously described for the detection of *Babesia* and *Theileria* species (Roy *et al*., 2000; Ano *et al*., 2001; Jefferies *et al*., 2003) and comparable to the assays developed by Birkenheuer *et al*. (2003a) and Fukomoto *et al*. (2001). Caution however should be taken in the interpretation of lowest detectable percentage parasitaemia due to the high variability of red blood cell counts (Birkenheuer *et al*., 2003a). The clinical sensitivity of PCR during pre-acute and chronic stages of infection for each of the canine piroplasm species has not been reported and would require further investigation using experimentally infected animals. Further study into the detection limits of PCR is described in Chapter eight.

5.5.2 Evaluation of the FTA® Classic Card DNA purification technique

The results of this study suggest that the use of FTA as a template for amplification of piroplasm DNA from canine blood is more sensitive than using QIAGEN and Isocode techniques. All three techniques were, however much less sensitive than using DNA extracted from whole blood (refer to section 5.4.1). DNA extraction using whole blood should be given priority over FTA-based methods to allow for increased levels of detection. Previous studies comparing both IsoCode™ Stix and FTA cards to store blood and diagnosis of malaria by PCR suggested that FTA cards showed the greatest level of sensitivity in the detection of mixed infections (Zhong *et al.*, 2001). Comparisons between DNA template from IsoCode™ Stix and QIAamp blood extraction techniques for the detection of pathogens in various sample types have also been previously conducted and suggested that the IsoCode™ Stix method was highly sensitive (Henning *et al.*, 1999; Coyne *et al.*, 2004).

The use of FTA cards as a method for transport, storage and DNA template for the PCR detection of pathogen DNA in blood samples has previously been suggested to be a highly efficient and sensitive technique (Zhong *et al.*, 2001; Subrungruang *et al.*, 2004). DNA of a *Babesia microti*-like parasite has also previously been amplified from blood sampling filter paper (Okabayashi *et al.*, 2001). While such studies have concentrated on the advantages of this method, such as the archival potential and high sensitivity, limited study has been carried on the possibility of cross-contamination between samples and the importance of sample preparation. The risk of cross-contamination between samples is considerable when using the suggested protocol by Whatman . The use of a single punch repetitively between samples offers a significant means of transferral of DNA between samples. Ultimately, the use of a new punch for each sample would be ideal, however the high cost (approximately AU$6 per punch) prohibits this. A modified technique based on the FTA punch cleaning protocol using sterile filter paper was devised in this study. This allowed for a significant decrease in cross-contamination and also included using of a negative control for each

sample tested. Further research should be carried out to optimise a suitable protocol with a risk of cross-contamination that is negligible.

The use of FTA paper for the application of arthropod samples for archiving and use for subsequent detection of pathogens using PCR has been previously reported for fire ants (*Solenopsis invicta*) (Snowden *et al*., 2002; Milks *et al*., 2004) and other arthropods (Bextine *et al*., 2004; Harvey, 2005). There may therefore be potential for using this technique for the storage and detection of piroplasm DNA in ticks.

5.5.3 Conclusion

This chapter has described the development of a simple nested PCR-RFLP technique for the detection and discrimination of the canine piroplasms. This assay has the potential to be implemented into a standardised screening protocol for *B. gibsoni* in dogs being exported from Australia and is evaluated in Chapter six. While FTA cards are potentially beneficial in regions where technology resources are limited, allowing for samples to be sent at an ambient temperature to a specialist laboratory, this study has shown that some limitations do exist, including the reduced detection limit and the risk of DNA cross-contamination. It was therefore decided that FTA was only to be used as a means of importing canine blood samples from overseas (refer to chapter ten). Further study needs to determine DNA purification methods from FTA cards that are comparable to DNA extraction from EDTA whole blood before this technique can be considered for routine diagnosis of infections.

CHAPTER SIX

Evaluation of PCR-RFLP for the screening of *B. gibsoni* infections in dogs being exported from Australia

6.1 Introduction

Following the first report of *B. gibsoni* infections in three American Pit Bull Terriers in the south eastern state of Victoria (Muhlnickel *et al.*, 2002), Australia has been defined as endemic for this infection by the Australian Quarantine and Inspection Services (AQIS). The risk of importing *B. gibsoni* into New Zealand (a country reportedly free from this pathogen) has been assessed by evaluating the import regulations which govern dogs travelling between the two countries and the likelihood of *B. gibsoni* infection becoming established (Beban, 2003). This lead to a change in screening protocols for dogs exported from Australia to New Zealand (http://aqis.gov.au/ accessed 9/2003).

Thus dogs that are to be imported into New Zealand from Australia must be tested at an AQIS approved laboratory for *B. gibsoni* infection according to the following schedule as specified by the Ministry of Agriculture and Forestry (MAF), New Zealand (http://www.biosecurity.govt.nz/imports/animals/standards/domaniic.aus.htm, accessed 12/ 2004):

i) Within 10 days from the scheduled date of shipment, a blood sample is collected for serum preparation and a thin blood smear made from a drop of blood obtained from the ear margin.

ii) The serum sample must test negative (cutoff is 1:40) to the indirect fluorescence antibody test (IFAT) for *B. gibsoni* using antigens appropriate

for the strains likely to be present in all the countries where the dog has been resident.

iii) The blood smear must be negative for *B. gibsoni*.

Dogs are also required to undergo acaricidal drug therapy before transport to New Zealand.

To further evaluate the nested PCR-RFLP assay described in Chapter five, a comparative study was conducted with microscopy and IFAT to assess this technique for screening *B. gibsoni* infections in dogs being imported into New Zealand from Australia.

6.2 Aim

- To evaluate the current *B. gibsoni* screening protocol for dogs being exported from Australia to New Zealand and compare it with PCR-RFLP based detection.

6.3 Material and Methods

6.3.1 Blood samples

Two hundred and thirty five blood samples (EDTA blood, serum and blood smears) were collected from dogs being screened as a requirement by AQIS and MAFNZ before being imported into New Zealand from Australia during 2003/04 (n = 229) or were submitted by Australian veterinarians due to suspected babesiosis (n = 6).

6.3.2 DNA extraction and PCR-RFLP

DNA was extracted from each EDTA canine blood sample according to the method described in Chapter four, section 4.2 and piroplasm DNA was amplified using the PCR conditions described in section 5.3.3. RFLP was used to discriminate between species (refer to Chapter five). Extracted DNA for each PCR negative sample was spiked with DNA of *B. canis vogeli* and then amplified to ensure the absence of PCR inhibitors.

6.3.3 Immunofluorescent Antibody Test (IFAT)

Immunofluorescent Antibody testing was performed using a modified procedure described by Anderson *et al*., (1980) (refer to Chapter four, section 4.9).

6.3.4 Light microscopy

EDTA thin blood smears were stained with a modified Wright-Giemsa stain using an Ames Hema-Tek® slide stainer (Bayer AG, Germany). Smears were examined according to the procedure described in Chapter four, section 4.1.

6.4 Results

Of the 235 blood samples screened, 11 were found to be positive for *B. gibsoni* using IFAT. One sample was microscopy positive and was also IFAT positive (Table 6.1).

	Microscopy +	Microscopy -	Total
IFAT +	1	10	11
IFAT -	0	224	224
Total	1	234	**235**

Table 6.1

Numbers of dogs positive for *B. gibsoni* infection using microscopy and IFAT

Four of the eleven IFAT–positive samples, were PCR-positive (Table 6.2). RFLP confirmed the presence of *B. gibsoni* in three of the samples and *B. canis vogeli* in the fourth sample. In addition, one sample was found to be PCR-RFLP positive for *B. canis vogeli* and was negative using both IFAT and microscopy. Seven samples were found to IFAT-positive and negative for both PCR and microscopy. Each of the IFAT negative samples were negative for *B. gibsoni* using both microscopy and PCR. Only one sample was positive for all three methods of detection.

	PCR-RFLP			
	***B. gibsoni* +**	*B. canis* +	***Babesia* -**	**Total**
IFAT +	3	1	7	11
IFAT -	0	1	223	224
Total	3	2	230	**235**

Table 6.2

Numbers of dogs positive for *B. gibsoni* and *B. canis* using IFAT and PCR-RFLP

Each of the IFAT-positive samples that were also positive for *B. gibsoni* by PCR had a titre that was 1 : 2560 or greater (Table 6.3).

IFAT titre	No of Samples	PCR
1 : 10240	2	Positive (*B. gibsoni*)
1 : 2560	1	Positive (*B. gibsoni*)
1 : 40	1	Positive (*B. canis*)
1 : 160	1	Negative
1 : 40	6	Negative
Total	**11**	

Table 6.3

Antibody titre values for IFAT positive samples and comparison with PCR results

Samples containing amplifiable *B. gibsoni* DNA on the basis of RFLP analysis were from two American Pit-bull Terriers from rural Victoria and one from an American Pit-bull Terrier cross from Sydney, New South Wales. RFLP results were supported by DNA sequencing (refer to Chapter ten). All PCR negative samples showed the absence of PCR inhibitors by spiking with *B. canis* DNA (Figure 6.1).

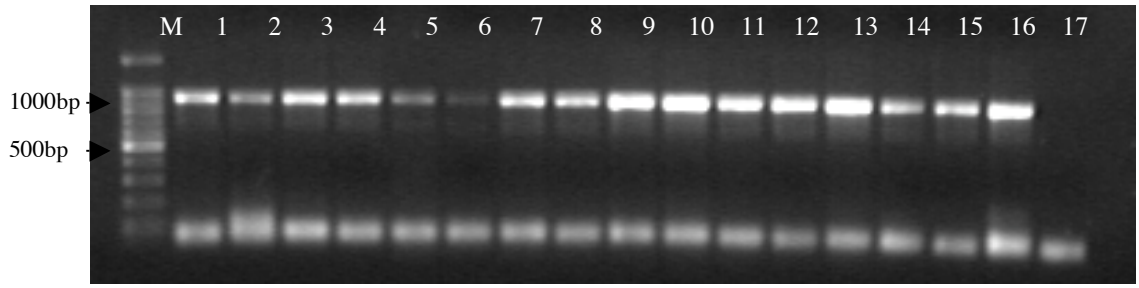


Figure 6.1

Spiking of PCR negative samples with *B. canis* DNA to test for PCR inhibition (M – molecular marker, 1 to 16 – spiked samples, 17 – negative control)

6.5 Discussion

While *B. gibsoni* infection has been reported in south eastern Australia, New Zealand is still suggested to be free from this parasite. It is reported that 73% of all dogs being imported into New Zealand are from Australia (Beban, 2003). Dogs being imported from Australia

therefore potentially pose a threat for the introduction of *B. gibsoni* to New Zealand. This threat is further supported by the results of this study. Using the current screening protocol recommended by MAF, New Zealand, eleven dogs would be considered positive for *B. gibsoni* using IFAT (cut-off titre 1:40). While it is theoretically possible that each of these dogs is positive, the fact that the only three that were also PCR-RFLP positive had significantly elevated titres (≥ 1 : 2560) supports the hypothesis that true *B. gibsoni* positive cases are indicated by a combination of high IFAT titre and PCR detection of parasite DNA. Only one of these dogs was positive using microscopy. Comparative analysis of PCR-RFLP, microscopy and IFAT for the detection of *B. gibsoni* infections has therefore highlighted the discordance that exists between different detection techniques.

Two major limitations exist with the current screening protocol. Firstly, all dogs being imported into New Zealand must have a blood smear that is negative for *B. gibsoni*, yet microscopy appeared to show a very low sensitivity in this study, a feature that is well recognised by previous research (Bose *et al.*, 1995; Krause *et al.*, 1996). Detection of *B. gibsoni* infections by microscopy can be useful during acute infections, when the parasitaemia is high. By contrast, microscopy fails to accurately detect *B. gibsoni* during chronic stages of infection when few parasites are found within the venous blood (Ano *et al.*, 2001). This important aspect of chronic *B. gibsoni* infections is investigated further in Chapter eight. Microscopy also fails to distinguish species and subspecies of *Babesia* and *Theileria* due to morphological similarity of these parasites (Conrad *et al.*, 1992). Detection is also limited by the experience of the microscopist due to the small parasite size and similarity of intraerythrocytic merozoites to nuclear remnants within red blood cells.

The second limitation relates to IFAT. Currently for export into New Zealand, dogs must have an IFAT negative serum sample (titre cut-off 1:40), however the results of this study suggest that cross-creativity of antigen can occur and may lead to the report of false positive results. One dog within this study was found to be PCR-RFLP positive for *B. canis vogeli*

and was also IFAT-positive (1 : 40). All other samples with an IFAT titre of 1 : 40 were found to be PCR-negative for piroplasm DNA and an additional PCR-negative sample gave a antibody titre value of 1 : 160. The cross-reactivity of antigen with other *Babesia* spp., and even parasites of other genera such as *Neospora* and *Toxoplasma* has previously been reported when using IFAT (Yamane *et al.*, 1993). Cross-reactions were reported for ten dogs naturally infected with *B. canis*, with eight giving a antibody titre less than or equal to 1 : 160 and two had titres of 1 : 320 (Yamane *et al.*, 1993). Increasing the currently accepted cut-off titre (to 1: 160) for *B. gibsoni* positive samples may help eliminate false positive cases and give a similar level of agreement to PCR, while retaining the high level of sensitivity of this detection technique. It has also been reported that IFAT may fail to detect dogs infected with *B. gibsoni* during early infections and that some dogs may fail to seroconvert (Farwell *et al.*, 1982). IFAT also fails to distinguish between current and previous infections. Further study is therefore necessary to determine the time taken to detect early *B. gibsoni* infections and titre levels post-infection using IFAT and is investigated in Chapter eight.

This study confirms that PCR-RFLP shows promise as an effective detection technique, as it is capable of detecting various *Babesia* species with a high level of sensitivity. Employing a technique that can detect *B. gibsoni*, in addition to multiple other species of canine piroplasm is beneficial in preventing other exotic species such as *T. annae, B. conradae* or the highly virulent *B. canis rossi* from entering New Zealand. It also enables dogs with *B. canis vogeli* infections that give an IFAT-positive result to be differentiated from those with actual *B. gibsoni* infections and preventing the unnecessary restriction of *B. canis* infected dogs. This is an important consideration, as Australia is known to be endemic to *B. canis vogeli* and a majority of dogs being imported into New Zealand are from Australia (Beban, 2003). PCR-RFLP was shown to be more sensitive than microscopy for the detection of *B. gibsoni* infections, a feature supported by previous studies (Bose *et al.*, 1995).

It is important to recognise that microscopy, IFAT and PCR-RFLP each have its own limitations. Microscopy was traditionally recognised as a 'gold standard' for diagnosing babesiosis, however this is no longer valid claim and a diagnostic test with levels of analytical sensitivity and specificity that are 100 % does not currently exist. It is therefore difficult to determine what constitutes a true positive and a true negative result. By understanding the benefits and limitations of current detection techniques, a combination of tests may offer the highest assurance for the detection of *B. gibsoni*, while minimizing the risk of producing false negative results. A revision of the current screening protocol for dogs being imported into New Zealand is therefore proposed and the use of combined IFAT and PCR-RFLP based detection is suggested (Table 6.4).

Recommendation	**Justification**
1. Removal of microscopic detection	Low sensitivity in detecting chronic infections
2. Replacement of microscopy with PCR-RFLP based detection	Higher sensitivity of PCR and the detection and differentiation of each of the reported canine piroplasm species
3. Raising of IFAT titre considered positive from 1 : 40 to 1 : 160	All PCR positive dogs had an IFAT titre of ≥1 : 2560. All PCR negative dogs had an IFAT titre < 1 : 160. One dog had an IFAT titre of 1 : 40 that was PCR positive for *B. canis vogeli*, suggesting antibody cross-reactivity and the risk of false positive results at this cut-off titre.

Table 6.4

Recommendations for changing the current protocol for screening dogs for *B. gibsoni* entering New Zealand

One PCR-RFLP and IFAT positive case was a dog from Sydney in New South Wales and had been proposed to be transported to New Zealand. The two remaining PCR-RFLP positive cases were of American Pit Bull Terriers from Victoria and were not being exported

from Australia. Additional research on *B. gibsoni* infections of dogs in Victoria is described in Chapter seven. While it is illegal to import dangerous dog breeds such as the American Pit Bull Terrier into New Zealand (http://www.biosecurity.govt.nz/imports/animals/standards/domaniic.aus.htm, accessed 2/2005), the possibility of other dog breeds being infected with *B. gibsoni* is significant. This is exemplified by the American Pit Bull Terrier–cross breed that was proposed to be exported from Australia to New Zealand being found to be positive for *B. gibsoni*. There have also been many reports in countries other than Australia of *B. gibsoni* infection in dogs of non-American Pit Bull Terrier breeds (Macintire *et al.*, 2002; Birkenheuer *et al.*, 2003b; Ikadai *et al.*, 2004).

The results of this chapter suggest that nested PCR-RFLP has the potential to be implemented into a standardised screening protocol for *B. gibsoni* in dogs being exported from Australia. A proposal for the change of current screening methods for dogs being exported from Australia to New Zealand, including the replacement of microscopic examination of thin blood smears with PCR-RFLP and increase of the IFAT cut-off titre, has been submitted to MAF, New Zealand[2] (Appendix A). This is also the first report of a *B. gibsoni* infected dog in New South Wales, extending the current known distribution of this pathogen in Australia.

[2] Subsequent to this investigation, a review of the current screening protocol was undertaken by MAF, New Zealand and a revision of the screening procedure is expected to be implemented late 2005.

CHAPTER SEVEN

Enzootic infections of *Babesia gibsoni* in American Pit Bull Terriers in south-eastern Australia

7.1 Introduction

An interesting feature of *B. gibsoni* infection is the high number of reports of this disease that have been described in fighting dog breeds such as the American Staffordshire Terriers and American Pit Bull Terrier in USA (Macintire *et al.*, 2002; Birkenheuer *et al.*, 2003b) and Tosa and American Pit Bull Terriers in Japan (Matsuu *et al.*, 2004; Miyama *et al.*, 2005). Studies of dogs from the Aomori Prefecture in Japan found that 3.9 % were positive for *B. gibsoni*, all were of the Tosa breed (Ikadai *et al.*, 2004) and 29.8% of all Tosa dogs studied from the same Prefecture were positive for *B. gibsoni* (Matsuu *et al.*, 2004a). A much broader study investigating suspected cases of *B. gibsoni* infection in 13 Prefectures throughout Japan found that 80 % of all positive dogs were Tosa and 11.4 % were American Pit Bull Terriers (Miyama *et al.*, 2005). Similarly, a high proportion (55 %) of fighting dog breeds were found to be positive for *B. gibsoni* in the southeastern United States (Macintire *et al.*, 2002). The significance of this breed predisposition to *B. gibsoni* infections is not yet fully understood, however the possibility of blood-to-blood transfer occurring between dogs during fighting has been speculated as a possible mode of transmission.

Within Australia to date, *B. gibsoni* has only been reported in the south-eastern state of Victoria in three American Pit Bull Terriers (Muhlnickel *et al*., 2002). Since then, no studies, have investigated the prevalence or transmission dynamics of this parasitic infection within Australia or determined if other dog breedshave been infected. This study investigates the prevalence and epidemiology of *B. gibsoni* in populations of American Pit Bull Terriers in Victoria subsequent to the initial case report.

7.2 Aims

i. To determine whether *B. gibsoni* infection had extended beyond the three dogs initially reported by Muhlnickel *et al*. (2002).

ii. If so, to determine the extent of *B. gibsoni* in American Pit Bull Terriers and other dog breeds within Victoria, Australia and if possible, to determine the prevalence of infection.

iii. To investigate possible modes of transmission of *B. gibsoni* among American Pit Bull Terriers.

7.3 Material and Methods

7.3.1 Dogs sampled[3]

EDTA blood and serum samples were collected from 151 dogs residing within the State of Victoria in south-eastern Australia during 2004/05. These included:

i. American Pit Bull Terriers from various rural properties between the towns of Warrnambool and Ballarat[4] (for the purposes of this study, termed non-show American Pit Bull Terriers) (n = 80). Nine of the dogs had blood collected on two occasions and one dog had three blood samples taken at separate times.

ii. Jack Russell Terriers and other dogs of non-American Pit Bull Terrier breed closely associated with (i) (n = 6).

iii. American Pit Bull Terriers at the annual show of the APBT Breeders Association, Melbourne (for the purposes of this study, termed show American Pit Bull Terriers) (n = 20).

iv. Non-American Pit Bull Terrier breeds from the same geographical locality as dogs in (i) that were patients of the Warrnambool Veterinary Clinic (n = 45) and were referred to as the control group of this study.

7.3.2 DNA extraction, amplification and RFLP

For each sample, DNA was isolated from 200 µl aliquots of EDTA blood (stored at -20 C) using a QIAamp® DNA mini kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions (refer to Chapter four, section 4.2). Amplification of a partial region of the 18S rRNA gene of *Babesia* spp. was performed as described in Chapter five, section 5.3.3. The species of piroplasm present was determined by RFLP analysis of the

[3] Ethics approval R1064/04

[4] The locality was chosen for investigation as one of *B. gibsoni* infected dogs described by Muhlnickel *et al.* (2002) resided in this district.

amplified product (refer to Chapter five, section 5.3.4) and further confirmed using DNA sequencing.

7.3.3 IFAT and microscopy

Antibodies to *B. gibsoni* were detected by IFA test (refer to chapter four, section 4.9) and a positive IFAT titre was considered to be 1:40. Thin blood smears were examined by microscopy for each sample (described in Chapter four, section 4.1).

7.3.4 Haematological data

The packed cell volume (PCV), red cell count (RCC), haemoglobin (HB), white blood cell count (WBC), platelet number (PLT) and total protein (TP) were determined for each of the blood samples using an ADVIA® 120 Haematology System (Bayer Healthcare LLC, Germany) and a Cell-Dyn 3500 haematology analyser (Abbott Diagnostics, U.S.A.).

7.3.5 Epidemiological data

Owners of each American Pit Bull Terriers involved in this study completed a questionaire (Appendix B) identifying the following epidemiological parameters:

i. Sex and age of the dog
ii. Kennelling information and whether the dog mixed readily with other dogs.
iii. Breeding history of the dog (i.e. – whether it had ever acted as a sire or breeding bitch).
iv. Interstate and overseas travel history.
v. Had the dog been witnessed being bitten by or biting another dog?
vi. Had the dog ever received a blood transfusion?
vii. Had ticks ever been found on the dog?
viii. Had any acarcidal treatments ever been given to the dog?

7.3.6 Statistical analyses

The Fisher's Exact Test, ANOVA and Mann Whitney test (SPSS v 12.0.1, SPSS, Chicago, IL) were used to assess statistical relationships between studied data. A kappa statistic was used to determine the level of agreement between PCR and IFAT results. Dogs that were less than six months of age were excluded from statistical analyses of haematology results due to differences exhibited between haematological values of young dogs and adults. A p value < 0.05 was considered statistically significant.

7.4 Results

7.4.1 PCR and IFAT screening for B. gibsoni

Of the 151 dogs studied, 14 were shown to be positive for *B. gibsoni* using either PCR, IFAT or both. All positive dogs were from the non-show American Pit Bull Terrier subgroup (Table 7.1).

			IFAT	
		No dogs positive	No dogs negative	Total
PCR	Positive	11	2	13
	Negative	3	55	58
	Total	14	57	71

Table 7.1

Comparison of PCR and IFAT detection of *B. gibsoni* infection in non-show American Pit Bull Terriers from Victoria

Simultaneous data for both IFAT and PCR was available for only 71 out of the 80 non-show Pit Bull Terriers studied. There was a strong agreement between IFAT and PCR for all dogs studied (kappa 0.90). Two PCR positive samples were IFAT negative and three IFAT positive samples were found to be PCR negative.

All show American Pit Bull Terriers and non-American Pit Bull Terrier breeds (associated dogs and all control group dogs) were negative for both detection techniques. *Babesia* parasites were not detected by microscopy in any dog during this sudy.

Of the 10 dogs that were sampled on multiple occasions, only one was consistently positive by both PCR and IFAT. Two dogs that were initially PCR/IFAT positive were treated with combined azithromycin and atovaquone, becoming PCR negative when tested approximately two months later but remaining IFAT positive. Two *B. gibsoni* positive female non-show American Pit Bull Terriers tested twice over a two-month period had pups. One that was consistently IFAT positive had five pups, two months of age and the other initially PCR/IFAT negative had three pups that were eight months of age at the time of sampling. All pups were negative for *B. gibsoni* using both IFAT and PCR.

RFLP results were confirmed by genetic sequencing, showing 100% homology with isolates from the United States and Japan (GenBank accession numbers AF271082, AF205636 and AF271081) on the basis of a partial region of the 18S rRNA gene (details of molecular characterisation are presented in Chapter ten).

7.4.2 Clinical signs and haematological data

Two of the *B. gibsoni* positive dogs had lethargy and bleeding from the mouth at the time of blood collection and were later euthanased. Unfortunately, no reliable clinical information or haematological data was available for these two dogs. All remaining dogs were found to be clinically normal at the time of blood collection, as examined by a veterinarian (Dr P. Irwin). Dogs that were positive for *B. gibsoni* had a significantly lower WBC ($p = 0.028$) and platelet count ($p = 0.002$) and a significantly higher total protein level ($p = 0.000$) than dogs that were infection-free (Table 7.2). All other haematological parameters were found to be normal for all dogs studied, whether infected or uninfected (raw data is shown in Appendix C).

		Mean value ± SD (No of dogs)	
	Total No.	***B. gibsoni* infection**	**Infection free**
WCC	55	9.4 ± 1.72 (7)	11.75 ± 2.65 (48)
PLT	55	197.57 ± 99.49 (7)	315.94 ± 90.5 (48)
Total protein	47	86 ± 10.86 (5)	73.62 ± 5.54 (42)

Table 7.2

Selective haematological parameters of non-show adult American Pit Bull Terriers with or without *B. gibsoni* infection.

7.4.3 Epidemiological data

i) Sex and age

Of all American Pit Bull Terriers studied (both show and non-show), 14/92 (84.8 %) were greater than 6 months of age and 21/97 (21.6 %) were bred by the owner of the dog. Of the non-show American Pit Bull Terrier males, 11/27 (40.7%) were significantly more likely to be infected with *B. gibsoni* compared to 3/39 (7.7%) females (p = 0.002).

ii) Kennelling and breeding history

Dogs that were individually kennelled (21.9%) were significantly more likely to be positive for *B. gibsoni* than dogs that were not individually kennelled (0%) (p = 0.011). No significance was found between *B. gibsoni* infection and being a sire or breeding bitch (p = 0.527).

iii) Travel history

Of all the American Pit Bull Terriers screened, 10/98 (6.7%) had previously travelled interstate and none had travelled overseas. None of the dogs that had travelled interstate or overseas were positive for *B. gibsoni*.

iv) Dog bite and history of blood transfusion

More non-show American Pit Bull Terriers that were reportedly bitten or were biters, were positive for *B. gibsoni* (12/41, 29.3%) than those not reportedly bitten (2/27, 7.4%) ($p = 0.027$). One dog that initially tested negative for *B. gibsoni* using PCR, later tested positive and had been reportedly attacked by another American Pit Bull Terrier. None of the dogs were reported to have had a blood transfusion.

v) Tick exposure and treatment

A single tick was found on one American Pit Bull Terrier by the owner subsequent to blood collection, however it was not available for species identification or PCR analysis. All other dogs were tick-free at the time of blood collection and had no reported history of tick exposure. None of the dogs had ever received acaricidal treatment.

7.5 Discussion

This study has demonstrated that *B. gibsoni* infection in Australia has occurred in more American Pit Bull Terriers than the three individuals initially described by Muhlnickel *et al.* (2002). Without testing many thousands of American Pit Bull Terriers in Australia, it is not possible to reliably determine the prevalence of this infection. Indeed, the prevalence within the American Pit Bull Terrier breed itself is likely to vary widely, a feature suggested by the results of this chapter, with a prevalence of 17.5 % among one group (non-show American Pit Bull Terriers) compared to the total absence of infection in the second group (show American Pit Bull Terriers). Thus for practical purposes, this study focussed on a discrete geographical region within Victoria from where one of the first cases originated (Muhlnickel *et al.*, 2002).

No other dog breeds were found to be infected with *B. gibsoni* and further research is necessary to investigate the possibility of this disease establishing within dogs other than American Pit Bull Terriers. Notably, the prevalence of this pathogen in non-fighting dog

breeds seems to be low in the United States (Macintire *et al.*, 2002; Birkenheuer *et al.*, 2003b) and all dogs found to be *B. gibsoni* positive from the Okinawa Prefecture were of various breeds that did not include either the Tosa or American Pit Bull Terrier (Ikadai *et al.*, 2004).

7.5.1 Detection of B. gibsoni infection

Both IFAT and PCR showed a general agreement for the detection of *B. gibsoni* in dogs within this study however, slight discordance was observed between these two detection techniques and all samples were microscopy negative. These results further support suggestions made in Chapter six, that accurate detection of *B. gibsoni* is problematic without an established 'gold standard' test and that microscopy exhibits a low level of sensitivity. The identification of two PCR-positive, IFAT-negative dogs and three PCR-negative, IFAT-positive dogs however, exposes the advantage of using a dual screening techniques approach. A possible explanation for the negative IFAT titre in two of these cases is that the infection was pre-acute and the dogs had not yet developed an immune response to the parasite. The failure of some dogs infected with *B. gibsoni* to seroconvert has also been reported (Farwell *et al.*, 1982). PCR-negative, IFAT-positive dogs may have been false-positive results as a result of antigen cross-reaction or true positive cases. Further study is therefore necessary to assess such cases, particularly the detection limit of PCR during chronic *B. gibsoni* infections, and this is investigated in Chapter eight. It is important to note that while all dogs were negative for *B. gibsoni* using light microscopy, this technique is generally the only detection method available to both veterinarians and commercial diagnostic laboratories, significantly limiting accurate diagnosis of this disease due to the low sensitivity and specificity of this method (Conrad *et al.*, 1991; Krause *et al.*, 1996). Detection using both PCR and IFAT has however been shown to be superior to microscopy (refer to Chapters five and six) and should be used when dealing with suspected cases of *B. gibsoni* infection.

7.5.2 American Pit Bull Terrier predisposition to B. gibsoni infection

The discovery of enzootic *B. gibsoni* infections in a population of American Pit Bull Terriers within Victoria and the absence of infection in other dogs breeds from the same locality, provides further support for the *B. gibsoni* infection predisposition of fighting dog breeds, such as American Pit Bull Terriers and Tosas reported in the USA and Japan (Macintire *et al.*, 2002; Birkenheuer *et al.*, 2003b; Ikadai *et al.*, 2004; Matsuu *et al.*, 2004a; Miyama *et al.*, 2005). The absence of infection in show American Pit Bulls and the presence of *B. gibsoni* in non-show dogs is likely to be consequence of different management practices by the owners of these dogs, a feature supported by analysis of questionnaire data. Perhaps the most important factor contributing to the disease within non-show American Pit Bull Terriers from rural localities between Warrnambool and Ballarat in Victoria is the increased risk of bitting or being bitten by another American Pit Bull Terrier and is further discussed with regard to transmission dynamics of *B. gibsoni* infection.

7.5.3 Transmission dynamics

It has been reported by Macintire *et al.* (2002) and Ikadai *et al.* (2004) that various modes of transmission may exist for *B. gibsoni,* contributing to the spread of this parasite. These include the role of direct blood contamination during dog fights, tick vectors, transplacental transmission and blood transfusion associated transmission. The first three of these mechanisms of transmission can be discussed with respect to the results of this study. The potential spread and increased distribution of this parasite can also be related to the movement of dogs from areas of endemicity to areas of non-endemicity.

i) Dog bite

This study supports the hypothesis of transmission occurring during dog fighting due to the high number of *B. gibsoni* positive dogs having been reportedly bitten by another dog. Dog fighting is illegal within Australia, however American Pit Bull Terriers are bred to attack other dogs, a situation that is likely to occur both accidentally and during illegal

'underground' dog fighting. Dogs often attack each other by biting the facial region of their opponent. Tentative evidence suggests that *Babesia* parasites are concentrated in the capillaries of its host (Breitschwerdt, 1984) and thus a higher concentration of parasite could be transmitted during mixing of facial capillary blood. Definitive evidence of blood-to-blood transmission of *B. gibsoni* would require controlled experimental fighting to occur between a positive and non-positive dog, a situation considered both un-ethical and illegal within Australia.

The first suggestion of possible direct blood-to-blood transmission of *B. gibsoni* between dogs, was made by Irizarry-Rovira *et al.* (2001), who reported *B. gibsoni* infection in a dog that had received multiple attack wounds from three American Pit Bull Terriers. The attack had occurred two months before the development of clinical signs and was consistent with the pre-patent period of *B. gibsoni* infection (Macintire *et al.*, 2002). A significant correlation between dog fighting and *B. gibsoni* infection has also been reported in Japan where the practice is still legal, with 26 of 35 positive dogs studied having been bitten by other dogs (Miyama *et al.*, 2005) and 47.1% of all dogs studied with a history of fighting being found to be positive (Matsuu *et al.*, 2004a).

ii) Tick vectors

The discovery of an unidentified tick species on only one American Pit Bull Terrier in Victoria, together with the absence of tick infestation reported by the dog owners suggests that ticks are potentially not significant in the transmission of *B. gibsoni* in the studies group of dogs from Victoria. Other studies have also suggested limited tick exposure in both American Pit Bull Terriers and Tosa. No ticks were identified on American Pit Bull Terriers infected with *B. gibsoni* in southeastern United States (Macintire *et al.*, 2002) and only three of 35 positive dogs had a confirmed history of tick exposure in a study in Japan (Miyama *et al.*, 2005).

The tick species *Haemaphysalis longicornis, H. bispinosa* and *Rhipicephalus sanguineus* have each been reported as possible vector candidates for the transmission of *B. gibsoni* (Swaminath and Shortt, 1937; Otsuka, 1974; Higuchi *et al.*, 1995), although definitive transmission studies using *R. sanguineus* have not been conducted. Within Australia, both *H. longicornis* and *R. sanguineus* are known to exist in Australia. *Rhipicephalus sanguineus* typically has a tropical to subtropical distribution, although has also been known to establish in more temperate locations if suitable conditions exist (Roberts, 1970; De Chaneet, 1976). *Haemaphysalis longicornis* was originally introduced from Japan to Australia in the early 1900's (Hoogstraal *et al.,* 1968) and it is now distributed throughout southeast Queensland (Sutherst and Bourne, 1991), coastal New South Wales, the Murray Valley and Western Australia (Besier and Wroth, 1985). Both tick species are potential vector candidates for *B. gibsoni* in Australia.

The involvement of ticks in disease transmission may however, be more significant than blood-to-blood transmission during dog fighting in certain countries and regions. It has been suggested that the main mode of transmission of *B. gibsoni* in the Okinawa Prefecture was likely to be by the tick *Rhipicephalus sanguineus* (Ikadai *et al.*, 2004). If *B. gibsoni* was to establish within regions of northern Australia, where dog infestation by *R. sanguineus* is extremely common, tick vector transmission may become more significant.

iii) Transplacental transmission

This study has identified two *B. gibsoni* infected female American Pit Bull Terriers that had infection-free pups. While it is likely that one of the adult females became infected with *B. gibsoni* after giving birth, the other was infected during pregnancy and transplacental transmission did not appear to occur or at least, patent infection was not maintained. A recent study however, proved experimental transplacental transmission of *B. gibsoni* and excluded the possibility of trans-mammary transmission (Fukumoto *et al.*, 2005). All pups died from acquired babesiosis. While vertical transmission of *B. gibsoni* can occur, this study

suggests it does not happen in all cases when the pregnant dog is known to be infected. The reason for case variation is unknown but could relate to the stage of infection and/or the immune status of the bitch.

iv) Movement of dogs to areas of non-endemicity

Only one dog residing outside of Victoria (located in Sydney, NSW) was reported to be infected with *B. gibsoni* (refer to Chapter six). The absence of reported cases in other Australian states, besides Victoria and New South Wales does not however, rule out the possibility of this parasite existing in other States and Territories of Australia. There are currently no restrictions on the movements of dogs throughout Australia and as this study has shown, dogs from enzootic regions have been reported to travel interstate.

New legislation has now been proposed for most states of Australia including New South Wales (http://dig.nsw.gov.au/dig/dighome/documents/circulars/05-20.pdf, accessed 5/2005) and Victoria (http://www.dpc.vic.gov.au/domino/Web_Notes/newmedia.nsf, accessed 4/9/2005), in which it will be an offence to breed, sell, give away or acquire Pit Bull Terriers and other similar breeds such as Japanese Tosas, Argentinean fighting dogs and Brazilian fighting dogs. All current owners of such breeds will also have to get their dogs de-sexed. Such banning of Pit Bull Terriers may help to limit the spread of *B. gibsoni*.

None of the dogs in this study had been reported to have travelled overseas, which together with current laws preventing the importation of American Pit Bull Terriers, make it difficult to speculate on the original source of *B. gibsoni*, in Australia. Reports suggest that *B. gibsoni* was likely to have first been introduced into the United States from military and/or fighting dogs being imported from Malaysia or Okinawa, Japan (Farwell *et al.*, 1982; Macintire *et al.*, 2002). Likewise, the initial introduction of this piroplasm into Australia may have also occurred as a consequence of importation of infected dogs and/or ticks from endemic countries such as Asia or the United States. Stringent control practices should now be put in

place to avoid further spread of this disease to non-endemic countries with screening protocols already existing for dogs being imported into New Zealand (http://www. biosecurity.govt.nz/imports/animals/ standards/domaniic.aus.htm, accessed 3/2005) and South Africa (http://www.aqis.gov.au.htm, accessed 3/2005).

7.5.4 Clinical signs of infections

It appears that most of the American Pit Bull Terriers described in this study had subclinical *B. gibsoni* infections, which further contributes to the difficulty of accurate clinical diagnosis. Indeed, B. gibsoni infection has previously been misdiagnosed as immune mediated anaemia (Muhlnickel *et al*., 2002). Two dogs, did however present with bleeding tendencies. This may reflect the thrombocytopenias found in most positive dogs, yet the platelet count would have to be severely depressed ($< 20 \times 10^9$/L) in order for bleeding to occur. Tosa dogs with subclinical *B. gibsoni* infections also had significantly lower mean platelet counts than dogs that were infection free. A significantly lower platelet count in *B. gibsoni* infected dogs was also reported in other studies (Macintire *et al*., 2002; Miyama *et al*., 2005).

7.5.5 Conclusion

This study has provided further evidence for the existence of *B. gibsoni* infections within Australia beyond the initial report of just three infected dogs. Increased veterinary awareness of these parasites, in addition to the employment of more effective detection methods such as PCR and IFAT, need to be considered if this infection is to be managed within Australia. While infected dog populations seem to be enzootic and restricted to dogs of American Pit Bull Terrier breed at present, the transmission potential to other dog breeds and other locations within Australia remains unknown. Careful management of known infected dogs, including antibabesial drug treatment and prevention of dog fighting is also necessary to help prevent the spread of this pathogen in Australia.

Babesia gibsoni infection should now be considered a significant disease of fighting dog breeds worldwide, is likely to be transmitted by direct blood exchange occurring during fighting/biting in these dogs and is a feature evident within American Pit Bull Terriers in Australia.

CHAPTER EIGHT

Experimental *Babesia gibsoni* infections: The assessment of combined atovaquone and azithromycin therapy and the detection limits of PCR during early and chronic stages of infection.

8.1. Introduction

While the *in vivo* culture of *B. gibsoni* has been reported previously in numerous studies (Anderson *et al*., 1980; Yamane *et al*., 1993; Wozniak *et al*., 1997; Wulansari *et al*., 2003; Matsuu *et al*., 2004), the clinical and pathological manifestations of these experimental infections seem to be varied and study dependent. Similar variation is also exhibited by natural infections (Irizarry-Rovira *et al*., 2001; Birkenheuer *et al*., 2003b; Matsuu *et al*., 2004a; Miyama *et al*., 2005). The disease can be pre-acute, acute, or chronic/subclinical. Acute infections are often typified by haemolytic anaemia, hemoglobinuria, thrombocytopenia and splenomegaly (Yamane *et al*., 1993). Chronic infections can develop, however this stage of infection can often be asymptomatic, with carriers of *B. gibsoni* infection acting as reservoirs of disease. Such subclinical carriers of infection are reported to maintain high antibody titres (Anderson *et al*., 1980; Farwell *et al*., 1982; Conrad *et al*., 1991; Yamane *et al*., 1993).

PCR has been shown to be highly sensitive and specific for the detection of *B. gibsoni* (Ano *et al*., 2001; Fukumoto *et al*., 2001; Jefferies *et al*., 2002; Birkenheuer *et al*., 2003a; Chapter five, sections 5.4.1, 5.4.2), yet limited study has been carried out on the detection limit of this technique during the early and chronic stages of infection. It is also not understood

whether canine piroplasms are capable of leaving the circulatory system of its host, to become sequestered within tissues such as the spleen.

Babesia gibsoni infection has a history of being notoriously difficult to treat successfully. Various treatments for *B. gibsoni* infection have been described (Farwell *et al.*, 1982; Wulansari *et al.*, 2003), however, no drugs have been reported to produce total eradication of circulating parasite. Atovaquone and azithromycin were first used as drug therapies for the eradication of malaria infections (Looareesuwan *et al.*, 1996; Taylor *et al.*, 1999) and were subsequently assessed as treatments for *B. microti* infections (Wittner *et al.*, 1996; Bonoan *et al.*, 1998). Combined drug treatment was found to be more effective than the use of each separately (Wittner *et al.*, 1996). While in some patients the drug therapy resulted in the total elimination of parasitaemia, other studies suggest that the clearance of parasites is inconsistent, with *B. microti* parasites sometimes persisting for months after treatment (Krause *et al.*, 2000).

A recent study assessed the efficacy of combined atovaquone and azithromycin therapy for the treatment of *B. gibsoni* and reported that the combined drugs reduced infections to undetectable levels (Birkenheuer *et al.*, 2004a). This study however did not use controlled experimental infections, but known naturally infected cases of chronic *B. gibsoni* infection in American Pit Bull Terriers. Some of the dogs given the treatment were found to remain PCR positive for *B. gibsoni* several months later, questioning whether this combined drug treatment is effective in all cases. A later report identified the possibility of drug resistence to atovaquone (Matsuu *et al.*, 2004b). Further study is therefore warranted to investigate the efficacy of combined atovaquone and azithromycin treatment in experimentally infected animals.

Experimental *B. gibsoni* infections were established in dogs for the production of IFAT blood slides, in addition to investigating the detection limits of PCR during various stages of infection and to assess the efficacy of combined azithromycin and atovaquone drug therapy.

8.2 Aims

i. To establish experimental *B. gibsoni* infections in dogs.

ii. To evaluate the efficacy of combined azithromycin and atovaquone drug therapy for *B. gibsoni* infections.

iii. To determine the detection limit of PCR during early and chronic stages of infection and compare with IFAT and microscopy.

iv. To assess the presence of *B. gibsoni* within various tissues when parasites have been eradicated from the bloodstream.

8.3 Materials and Methods

8.3.1 B. gibsoni positive blood samples

Babesia gibsoni-infected blood was collected from naturally infected American Pit Bull Terriers (refer to epidemiological study in Chapter seven). Blood samples were mixed into CPD-1 solution (Baxter International Inc., USA) and refrigerated at 4 C. Samples were screened for the presence of *B. gibsoni* using PCR-RFLP. DNA sequencing was later used to confirm the species and genotype of canine piroplam present (refer to Chapter four, section 4.7).

Infected blood was then passaged into experimental Dogs A and C, while Dog B received infected blood from Dog A (refer to section 8.3.3).

8.3.2 Experimental dogs[5]

Three five-month old, female beagle foxhound-cross dogs (Dogs A, B and C) were obtained from the Commonwealth Serum Laboratories, Melbourne. Each dog was sprayed with Frontline (2.5 g/L fipronil) (Merial, France) as a tick control measure before the initiation of the experiment and also once during the course of the experiment. Blood was collected at the initial time of arrival in Perth and screened using PCR and IFAT to confirm the absence of *Babesia* parasites. Dogs were housed indoors in the animal isolation facility at Murdoch University and examined daily.

8.3.3 Experimental infection with B. gibsoni (overview)

Immediately prior to passage of infected blood, a 5 ml blood sample was collected from the recipient dog for serology, PCR and haematological analysis. Passage details for each dog are described in Table 8.1. Each dog had 1 ml EDTA and 4 ml clotted for serum was collected daily for the duration of the experiment and a further 4 ml of clooted blood for

[5] Ethics approval R1063/04

serum was taken daily for the first 3 weeks of the experiment and then every 5 days. Rectal temperature, pulse rate, respiratory rate and general physical condition were also assessed daily for the duration of the experiment.

Experimental Dog	Passage details	Duration of Experiment	Comments
A	7 ml of blood from a naturally infected American Pit Bull Terrier (parasitaemia was not calculated since *B. gibsoni* could not be visualised).	70 days	An additional blood sample was taken 15 hrs post-passage
B	9 ml of blood from Dog A on day 27 post-passage (parasitaemia = 1.51 %).	121 days	Additional blood samples were taken at 1, 6 and 24hrs post-passage
C	8 ml of blood from a naturally infected American Pit Bull Terrier (parasitaemia was not calculated since *B. gibsoni* could not be visualised).	78 days	Additional blood samples were taken at 1, 6 and 24hrs post-passage

Table 8.1

Passage details for Dogs A, B and C

8.3.4 Haematological analysis of blood samples

EDTA blood samples were submitted for daily automated haematological analysis (CBC/DIFF) using an ADVIA® 120 Haematology System (Bayer Healthcare LLC, Germany). Haematological data were calculated for white blood cell count (WBC), red blood cell count (RBC), haemoglobin (HGB), haematocrit (HCT), mean cell volume (MCV), (MCH), (HCHC), (CHCM), (CH), (RDW), HDW, platelet number (PLT), (MDV), neutrophil number (#NEU), lymphocyte number (#LYMPH), monocyte number (#MONO), eosinophil number (#EOS), basophil number (#BASO) and leucocyte number (#LUC).

Biochemical analysis (serum: panel, electrolytes) was conducted on serum from Dog B, day 101.

8.3.5 Microscopy, IFAT and PCR

Thin blood smears were prepared from each EDTA blood sample and stained with a modified Wright-Geimsa stain using an Ames Hema-Tek slide stainer (Bayer AG, Germany). Percentage parasitaemia was calculated daily according to the method described in Chapter four, section 4.1. DNA was extracted from 200 µl of EDTA blood according to Chapter four, section 4.2. DNA was then amplified using nested-PCR (refer to Chapter five, sections 5.3.1, 5.3.3). *Babesia gibsoni* serology was conducted by IFAT on serum samples according to the method described in Chapter four, section 4.10.

8.3.6 Drug treatment of experimental B. gibsoni infection

Combined azithromycin and atovaquone drug therapy was administered to each of three experimental dogs. Azithromycin (Zithromax, Pfizer Ltd) and atovaquone (Wellvone, Glaxosmithkline) were used at the dosage rates given by Birkenheuer *et al.* (2004a). Each drug was given for ten days and administered orally (Table 8.2). A high fat meal was given to each dog following drug administration to assist with the intestinal absorption of atovaquone. The time of initiation of therapy was dog dependent (Table 8.2).

Dog	Weight (kg)	Initiation of drug therapy	Stage of infection	Azithromycin[1] 10 mg/kg q 24 h PO	Atovaquone[2] 13.3 mg/kg q 8h PO
A	13.3	Day 31	Acute	3.3 ml	1.2 ml
B	11.36	Day 53	Acute	2.84 ml	1 ml
	11.3	Day 95	Chronic	2.84 ml	1 ml
C	12.1	Day 52	Chronic	3.03 ml	1.06 ml

1. Zithromax® (Pfizer Ltd, Australia), 200mg/ml
2. Wellvone® Suspension (Glaxosmithkline, Australia), 500mg/ml

Table 8.2

Dosage rates for azithromycin and atovaquone administered to Dogs A, B and C (q =quaque, PO = per os).

8.3.7 Collection of saliva, urine and tissue samples

Saliva was collected by syringe from Dog A on day 25 and Dog B on day 51 and also by placing FTA paper directly into the mouth to absorb any saliva present. DNA was extracted from the saliva collected using a syringe using the QIAGEN blood extraction protocol (Chapter four, section 4.2). Saliva collected on FTA was allowed to dry and then purified according to the FTA purification procedure (Chapter five, section 5.3.7). Dogs A, B and C were euthanised by intra-venous barbiturate overdose on days 70, 121 and 78 respectively. At necropsy, each dog had tissue samples collected from spleen, pancreas, kidney, heart, lymph node, salivary gland, bone marrow, skin and intestine using a new scapel blade for the collection of each tissue. Tissues were stored separately A urine sample was also taken by cystocentesis. DNA was extracted from tissue samples using a QIAamp DNA mini kit (QIAGEN, Germany), according to the tissue protocol (refer to Chapter four, section 4.3). DNA was extracted from urine using a QIAamp DNA mini kit according to the protocol (refer to Chapter four, section 4.2) for blood and body fluids.

8.3.8 Cryopreservation of canine blood infected with B. gibsoni.

For archival purposes, blood samples from Dog A were collected on day 23 and for Dog B on days 52 and 121 were cryopreserved according to Dalgliesh (1971). An equal volume of 4M dimethyl sulphoxide (DMSO) in PBS was added to blood. Half a ml of cold DMSO was added to 0.5 ml cold blood at a rate of 1 ml/30 sec, gently mixed and then held at 4 C for 10 min, -20 C for 20 min and –80 C for 20 min. The blood was then transferred to liquid nitrogen for long-term storage.

8.3.9 Statistical analysis

Statistical analysis was performed using SPSS v12.0.1 (SPSS, Chicago, IL). Correlations between parasitaemia and haematological data were determined using a Pearson correlation test and a p-value less then 0.05 was considered significant.

8.4 Results

8.4.1 Clinical observations

Each dog was successfully infected with *B. gibsoni*. Only two dogs (Dog A and B) exhibited clinical signs of infection. Dog A developed loss of appetite on days 19-23 and fever on days 23-28. A significant positive correlation was observed between parasitaemia and rectal temperature ($p = 0.000$) and heart rate ($p = 0.015$) for Dog A. Mild icterus was observed on days 26-28. Splenomegaly developed in Dog B on day 52 and slight icterus was also observed on day 55. Dog C never developed clinical signs of infection. No significant side effects were observed during the periods of drug therapy for each of the three dogs, although fatigue and excessive salivation were witnessed for Dog B during the second course of drug therapy. Subsequent biochemical analysis revealed no abnormality.

8.4.2 Microscopic detection of B. gibsoni

For the purposes of this study, the early stage of infection was defined to be from the time of passage to the first detection of *B. gibsoni* by light microscopy. In both dogs A and B,

distinct, pre-acute, acute and chronic stages of infection could be differentiated during the course of experimental infection (Figure 8.1). Early infection was determined to be from day 0-6 for Dog A and day 0-36 for Dog B, although one parasite was microscopically visualised on day 5 (Figure 8.1 i and ii). The acute stage of infection was considered as the time during which *B. gibsoni* parasites could be detected by microscopy and was from day 7-31 for Dog A, reaching a maximum of 1.5 % parasitaemia on day 27. Drug treatment was initiated on day 31 when parasitaemia had dropped to 0.875 % and no parasites were observed subsequently. Two acute stages of infection were observed for Dog B on days 37-60 and 107-121 (Figure 8.1 ii). The level of parasitaemia reached a maximum of 6.02 % on day 51. Drug treatment was initiated on day 53 when parasitaemia showed a slight decrease to 5.82 %, then a rapid decrease in parasite number until day 60 when no parasites were observed.

The chronic stage of infection was from day 33-70 for Dog A and day 61-106 for Dog B (Figure 8.1 i and ii). Dog C failed to develop a parasitaemia detectable by microscopy and was therefore deemed to have a chronic infection for the duration of the experiment (Figure 8.1 iii).

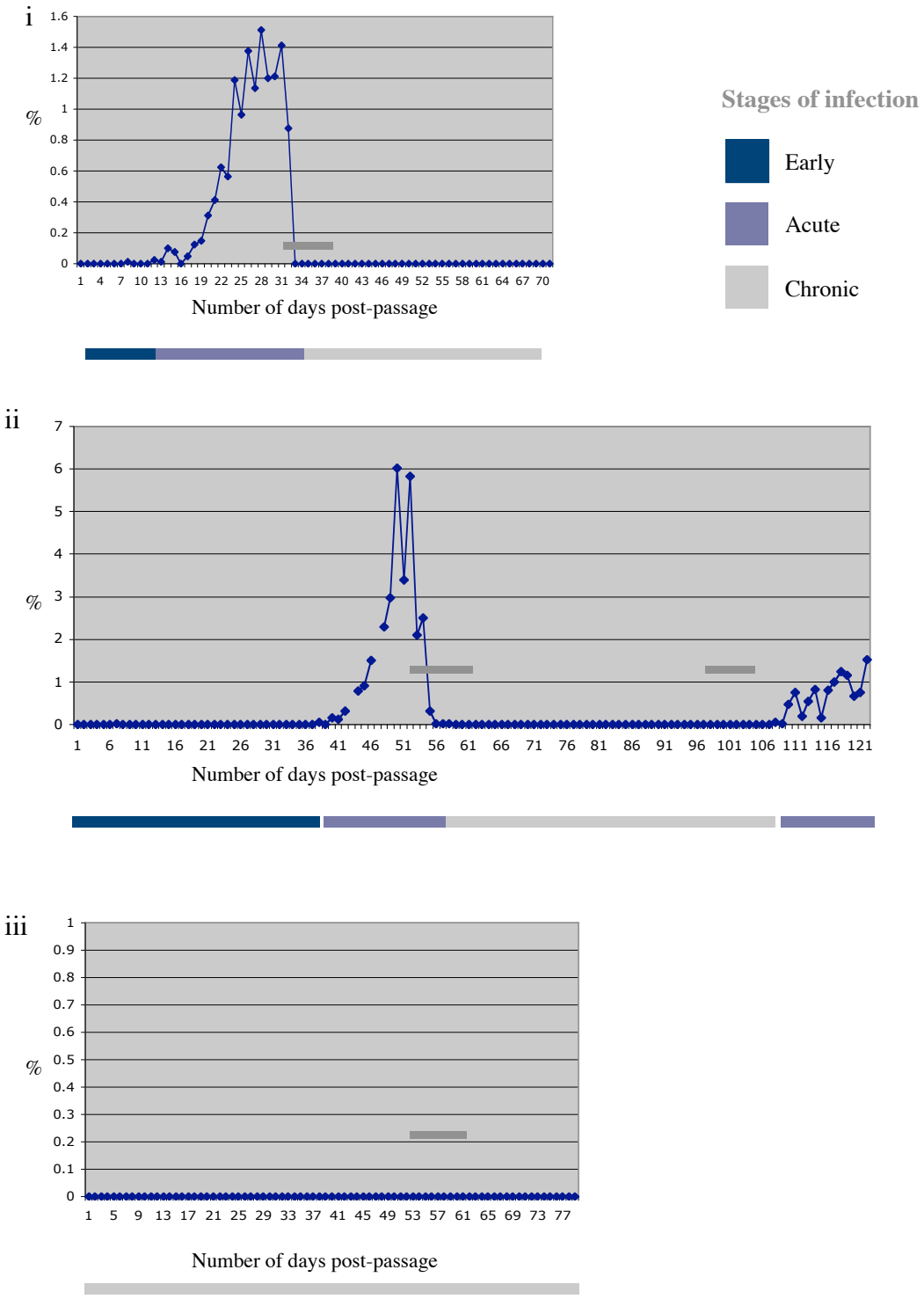


Figure 8.1

Parasitaemia levels ■ (%) for Dogs A (i), B (ii) and C (iii). Dark grey bars indicate periods of drug treatment.

Morphological changes were observed for intraerythrocytic merozoites of *B. gibsoni* during the initial acute stage of infection for Dog B, pre- and post-drug therapy (Figure 8.2).

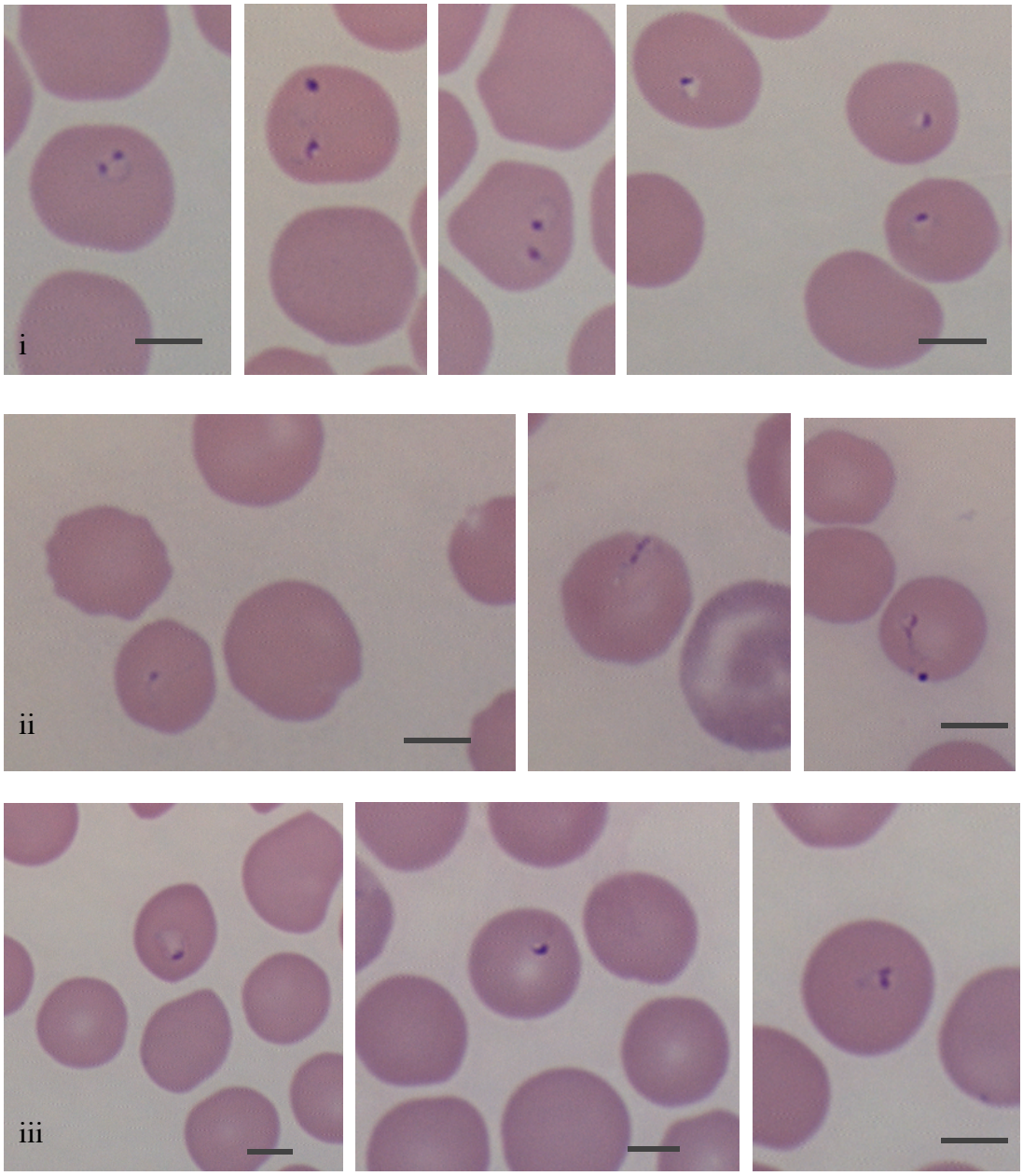


Figure 8.2

Morphological variation of *B. gibsoni* merozoites from Dog B before drug treatment on day 52 (i) and post treatment on days 57 (ii) and 119 (iii). Scale bar represents 5 □m.

Before the first period of drug treatment, parasites appeared as singular or paired intra-erythrocytic merozoites (Figure 8.2 i). They were polymorphic and ranged in size from 1 m to 3 m in diameter. After the initiation of drug therapy, merozoites became smaller, with the absence of distinct cytoplasmic inclusions (Figure 8.2 ii). In some cases, nuclear material appeared degraded and developed into extended stands within the erythrocyte. While parasites could not be detected by microscopy during the second period of drug treatment for Dog B, merozoites were observed showing typically morphology and the presence of dividing forms two days after the completion of the drug therapy (Figure 8.2 iii).

8.4.3 Haematology

A statistical correlation between RBC, HGB and HCT with parasitaemia was observed for both Dog A ($p = 0.000, 0.000, 0.000$) and Dog B ($p = 0.008, 0.026, 0.001$). Red blood cell count decreased with increasing parasitaemia and reached the lowest level as parasitaemia decreased to a level almost undetectable by microscopy (Figure 8.3).

A statistical correlation was also observed between PLT and MPV with parasitaemia was also observed for Dog A ($p = 0.000, 0.000$) and Dog B ($p = 0.000, 0.000$). Platelet number decreased with increasing percentage parasitaemia however, reached the lowest level during the early acute stage, before percentage parasitaemia peaked (Figure 8.4). All other haematological parameters were within a normal range.

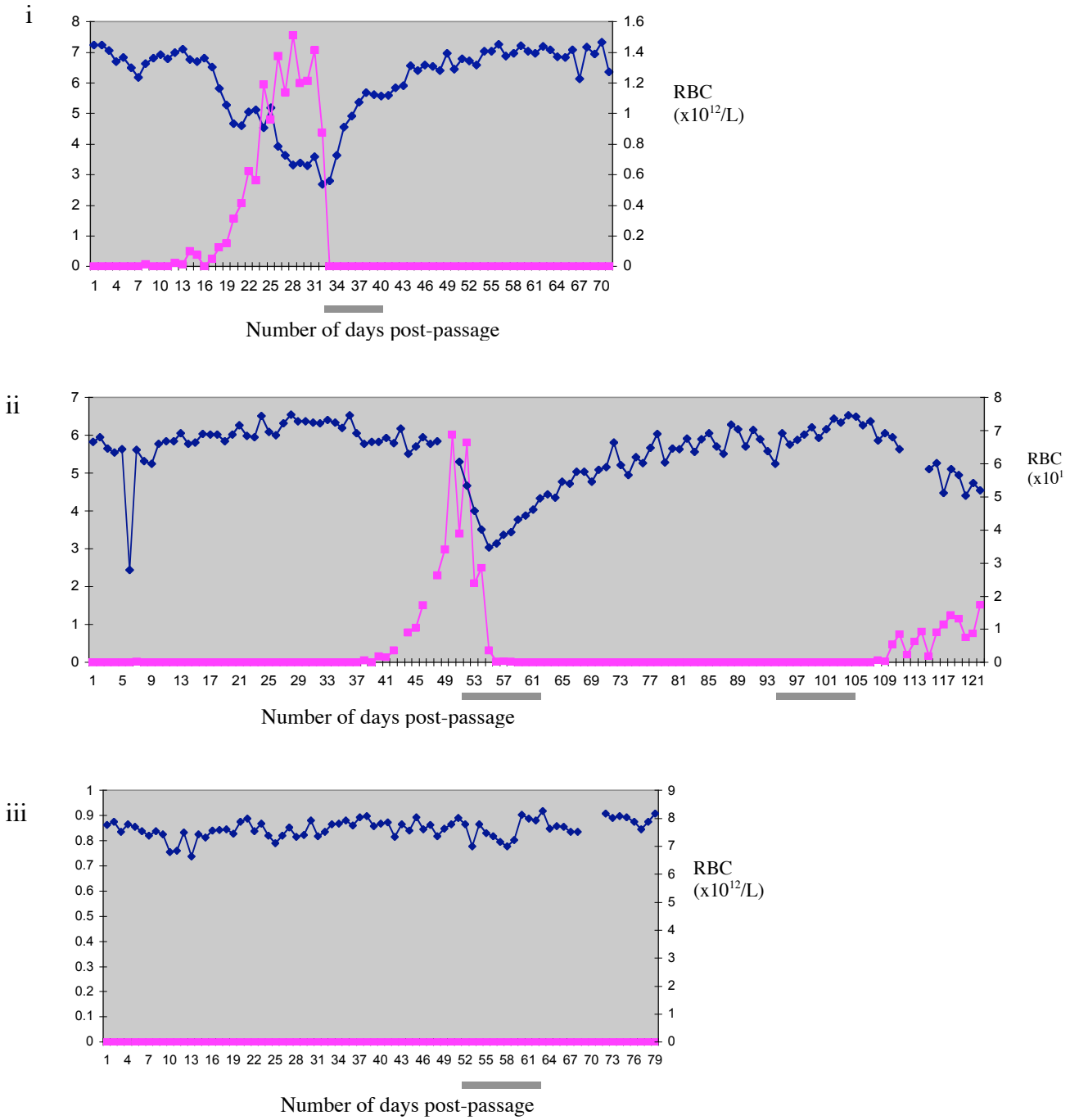


Figure 8.3

Plot of red blood cell number (x10^{12}/L) and parasitaemia (%) after initial passage of *B. gibsoni* over 70 days for dog one (i), 121 days for dog two (ii) and 78 days for dog three (iii). Grey bars represent periods of drug treatment

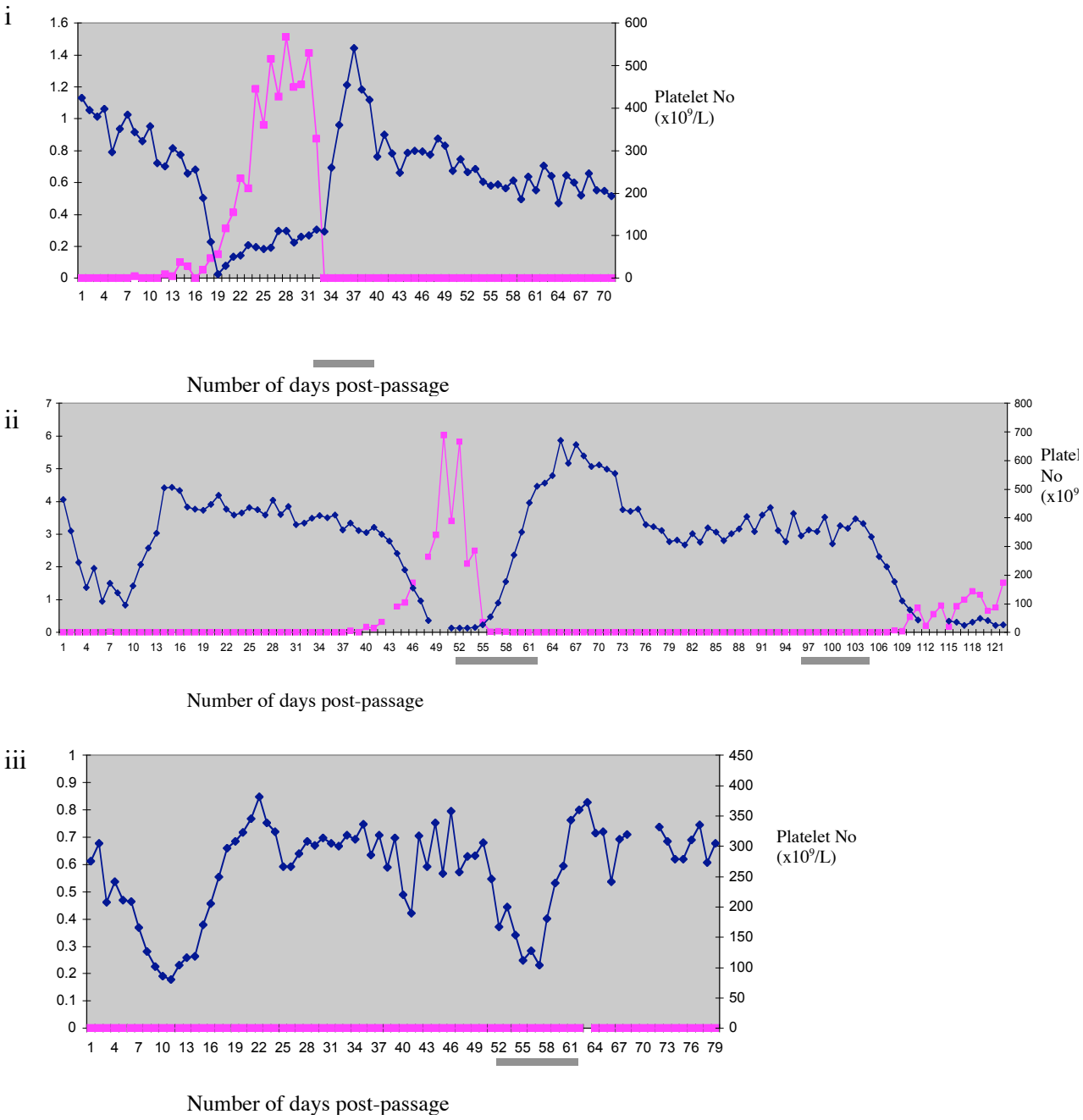


Figure 8.4

Plot of platelet number (x10^9/L) and parasitaemia (%) after initial passage of *B. gibsoni* over 70 days for dog one (i), 121 days for Dog Two (ii) and 78 days for Dog Three (iii). Grey bars represent periods of drug treatment.

8.4.3 Detection of B. gibsoni using IFAT

Each dog gave a negative IFAT result before passage of *B. gibsoni*. During the early and acute stages of infection, a positive IFAT titre of 1:160 was recorded for days 1-9, then a reduced titre of 1:40 for days 10-13 before increasing to a maximum of 1:10240 on day 19 for Dog A (Figure 8.5 i). Dog B was IFAT positive on day one (1:40) and remained positive with an increasing titre, reaching a maximum of 1:10240 on day 40 (Figure 8.5 ii).

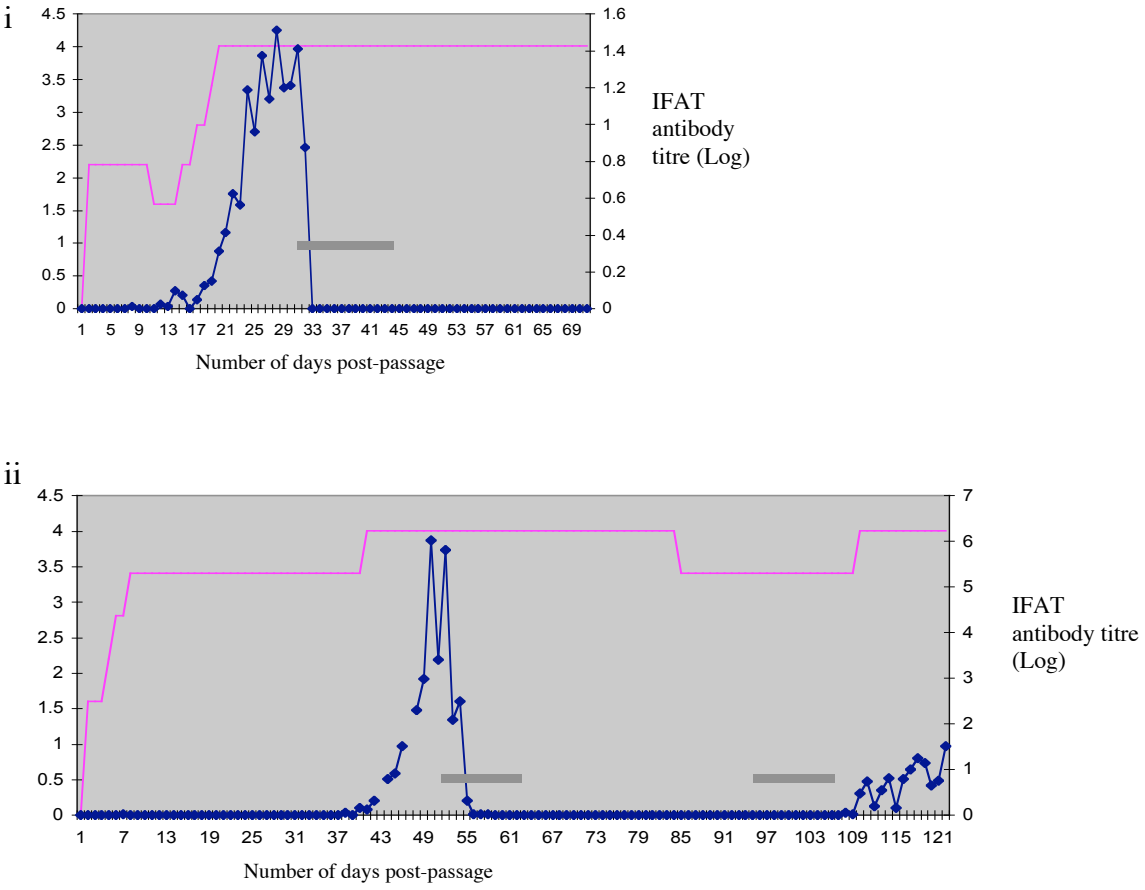


Figure 8.5

Plot of IFAT antibody titre ----- (Log) and parasitaemia ■ (%) during experimental infection for Dog A (i) and Dog B (ii). Dark grey bars indicate periods of drug therapy.

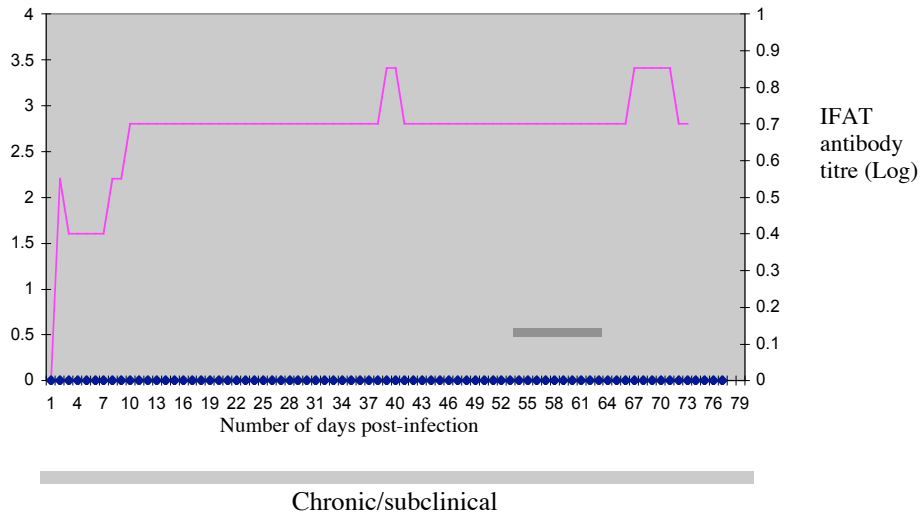


Figure 8.6

Plot of IFAT antibody titre ----- (Log) and parasitaemia ■ (%) during experimental infection for Dog C. The dark grey bar indicates period of drug therapy.

An IFAT titre of 1:10240 was maintained for the duration of the chronic phase of infection for Dog A, however Dog B showed a decreasing titre (lowering to 1:2560 On day 84) during this period before increasing to a maximum of 1:10240 with the onset of the second acute stage of infection. Dog C was positive by IFAT for *B. gibsoni* on day one (1:160), with elevated titres observed for the duration of the experiment (Figure 8.6). The number of days taken to reach the titre values, 1 : 40, 1 : 160 and 1 : 640 is shown in Table 8.3.

	Days taken to reach titre value		
Dog	**1 : 40**	**1 : 160**	**1 : 640**
A	1	14	16
B	1	5	6
C	1	7	9

Table 8.3

Time (days) taken to reach individual antibody titre values for Dogs A, B and C.

8.4.4 Detection of B. gibsoni by PCR during early and acute stages of infection

DNA of *B. gibsoni* was amplified in the secondary round PCR 15 hours after experimental passage of Dog A and was amplified one hour after passage for Dog B (Figure 8.7) and Dog C. The infected blood inoculated into Dog B had a 1.51% parasitaemia and contained an estimated 5.65 x 10^7 infected red blood cells. DNA was amplified from approximately 10 008 infected cells from Dog B after 1 hr. The detection limit for Dogs A and C within the first 1-15 hrs could not be definitively calculated as a parastiaemia could not be determined for the donor dogs.

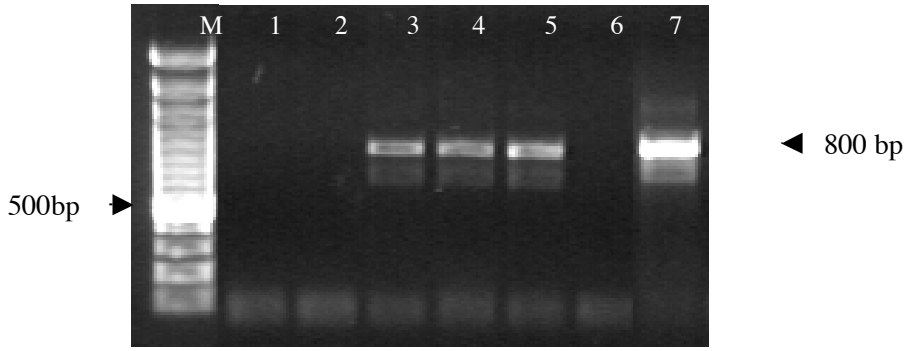


Figure 8.7

Secondary round PCR products amplified from blood taken from Dog B (M – 100 bp Molecular marker, 1 – day previous to passage, 2 – immediately pre-passage, 3 – 1 hr post-passage, 4 – 6 hr post-passage, 5 – 24 hr post-passage, 6 – negative control, 7 – positive control)

DNA was not detected in the primary PCR until day 6 for Dog A and day 2 for Dog B. For Dog B, DNA was only detected in the secondary PCR on day 8 and was not detected in the primary round again until day 29 for Dog B. PCR detected *B. gibsoni* consistently during the acute phase of infection for both Dog A and B.

8.4.5 PCR detection during chronic/subclinical stages of infection

DNA was amplified only in the secondary round for Dog C for the first 9 days post-passage (Figure 8.8).

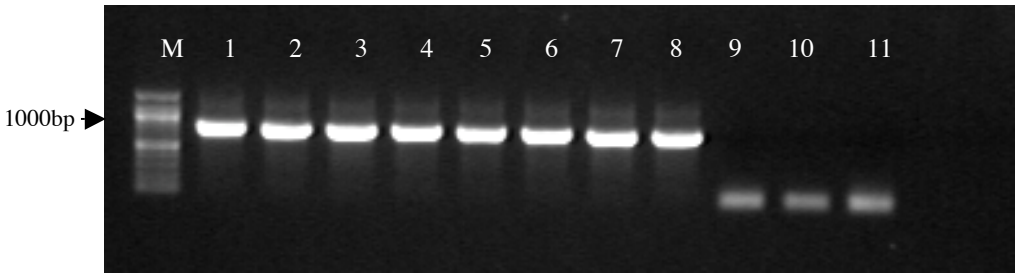


Figure 8.8

Secondary round PCR detection of *B. gibsoni* for Dog C (M – molecular marker, 1 to 11 – days 2 to 12).

No DNA was detected using PCR from day ten until day 20 when subsequent detection was only intermittent. Detection of DNA in the primary round PCR was only observed on days 63 and 78 for Dog C. The detection of *B. gibsoni* DNA within venous blood samples became intermittent post-drug therapy for Dogs A and C (Figure 8.9), however was consistently detectable for Dog B during the entire duration of the experimental infection.

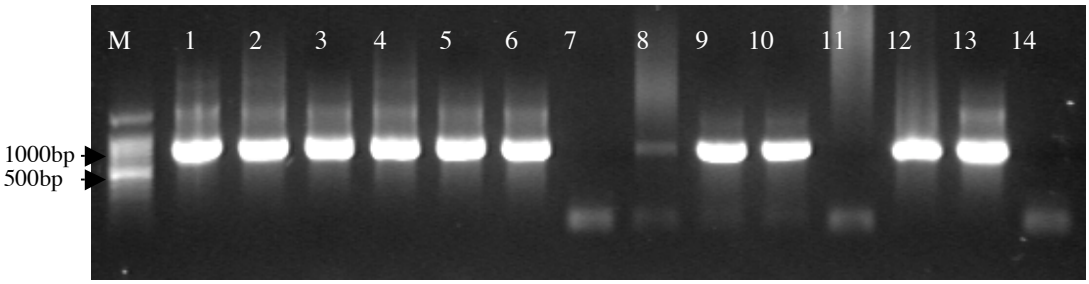


Figure 8.9

Intermittent detection (secondary PCR) of *B. gibsoni* DNA during chronic stage of infection for Dog C (M – molecular marker, 1-13 –days 54 – 66, 14 – negative control)

The average number of days PCR positive during chronic infection was determined for each of the dogs (Table 8.3). The lowest detection rate was found in Dog A, in which PCR detected *B. gibsoni* DNA only 43.59 % of the time. This is approximately equivalent to three positive days per week.

Dog	**No of PCR negative days**	**No of PCR positive days (%)**	**Total**
A	22	17 (43.59)	39
B	0	49 (100)	49
C	24	54 (69.23)	78

Table 8.3

PCR detection of *B. gibsoni* during chronic/subclinical stages of experimental infection

8.4.6 PCR detection of B. gibsoni *DNA in tissue, urine and saliva samples*

DNA of *B. gibsoni* could not be detected in the saliva samples taken during the acute stage of infection for Dogs A and B. Likewise salivary gland tissue taken at post-mortem was also PCR negative for *B. gibsoni* for each of the three dogs. The urine sample for Dog C was positive for *B. gibsoni*, while urine from Dogs A and B was PCR negative.

Babesia gibsoni DNA was amplified in lymph node tissue from dog one, brain, kidney, spleen, heart, lung, salivary gland and skeletal muscle for Dog B and in spleen for Dog C (Figure 8.10).

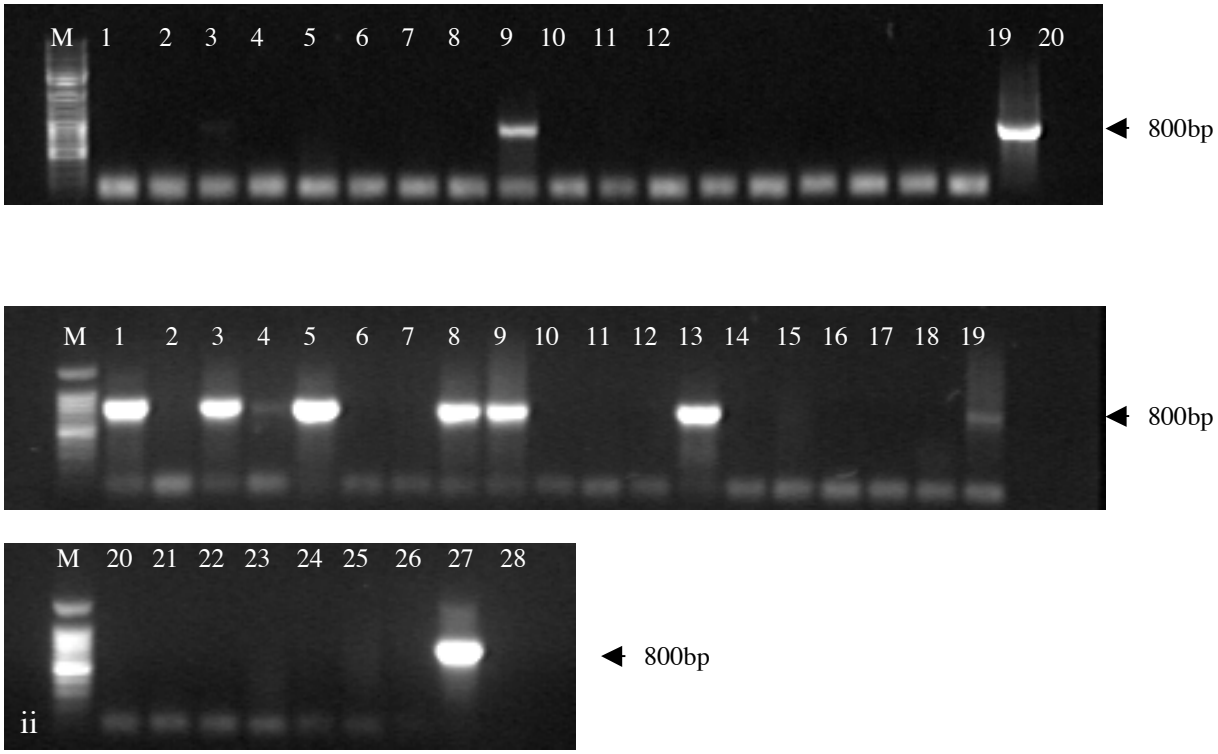


Figure 8.10

M – molecular marker, i) Dog A (1-spleen, 2-pancreas, 3–kidney, 4–salivary gland, 5–liver, 6–lung, 7–skin, 8–heart, 9–lymph, 10–bone marrow, 11–small intestine, 12–urine, 19–positive control, 20–negative control). ii) Dog B (1–brain, 2–Retropharyngeal lymph node, 3–kidney, 4–lung, 5–spleen, 6–tonsil, 7–mesenteric lymph node, 8–heart, 9–Salivary gland, 10–liver, 11–intestine, 12-adrenal, 13-skeletal muscle) and Dog C 14 - brain, 15 –Retropharyngeal lymph node, 16 –kidney, 17–lung, 18–tonsil, 19–spleen, 20–mesenteric lymph node, 21–heart, 22–Salivary gland, 23–liver, 24–intestine, 25-adrenal, 26 -skeletal muscle) 27 – positive control, 28 – negative control.

8.5 Discussion

Successful experimental *B. gibsoni* infections were established in this study and in two cases were passaged using blood from chronically infected American Pit Bull Terriers that were microscopically negative for *B. gibsoni*. Highlighted by this investigation, are both the variability of infection and the difficulty in detecting *B. gibsoni* during early and chronic infections.

8.5.1 Clinical and clinicopathological indicators of infection

The variable clinical signs and clinicopathological parameters exhibited by each of the experimental dogs are similar to findings in previous studies suggesting some dogs develop a rapid, acute disease characterised by intermittent fever, thrombocytopenia and haemolytic anaemia, while other cases can be asymptomatic (Meinkoth *et al*., 2002; Matsuu *et al*., 2004a). In two of the experimentally infected dogs, a sudden decline in platelet number could be correlated to a rapid rise in parasitaemia. Thrombocytopenia has been reported in many cases of both experimental and natural *B. gibsoni* infections (Macintire *et al*., 2002; Miyama *et al*., 2005). This has been explained as an immune-mediated mechanism in which IgG binds to the platelet surface, resulting in the removal of platelets from circulation and a decrease in the mean platelet volume (Wilkerson *et al*., 2001; Matsuu *et al*., 2004a) or as a result of excessive release of inflammatory mediators during the process of erythrocyte lysis (Lobetti, 1998).

One dog (C) developed a subclinical infection for the entire duration of the experiment and failed to exhibit any physical signs of infection, exposing the unreliability of clinical signs in diagnosing *B. gibsoni* infections. No parasites were observed by light microscopy further highlighting the limitation of this detection method during early and chronic infections. Numerous reports describe the low detection limit of microscopy and its inabaility to accurately differentiate species, both of which are discussed in detail in Chapter five, section 5.4.2.

8.5.2 Combined atovaquone and azithromycin drug treatment

While combined atovaquone and azithromycin produced a rapid reduction in the number of circulating parasites, it is probable that *B. gibsoni* was not totally eradicated in any of the experimental dogs. The results of this experiment also suggest that in certain cases *B. gibsoni* can develop resistance to the drugs atovaquone and azithromycin. This was reflected in one of the experimental dogs, which showed an increasing circulating parasitaemia after a second period of drug treatment. Atovaquone resistance has also been reported in other studies and has been found to be associated with point mutations within the cytochrome b gene of *B. gibsoni* (Birkenheuer and Marr, 2005; Matsuu *et al*., 2005). A single nucleotide mutation in the cytochrome b gene, resulting in one amino acid replacement, was found in parasites after atovaquone treatment but was not present in parasites pre-treatment (Matsuu *et al*., 2005). Sequencing of the cytochrome b gene of isolates pre- and post-drug therapy from the experimental dogs described in this chapter would allow for confirmation of resistance. Further study into more effective drug therapies is warranted and multiple novel drugs are currently being investigated as possible curative treatments for *B. gibsoni* infection, including compounds derived from plant extracts from Indonesia (Subeki *et al*., 2005) and Africa (Naidoo *et al*., 2005).

8.5.3 Detection limits of PCR and IFAT in early and chronic infections

Early and chronic infections with *B. gibsoni* are difficult to detect using traditional methods, a feature which is of clinical relevance in natural cases of infection. For example, a recently infected dog in Australia may be negative by both microscopic examination and IFAT, thus escaping detection using these tests prior to export to New Zealand (refer to Chapter six). To date, there have been no studies published that have investigated the dynamics of PCR detection in these important stages of *B. gibsoni* infection.

i) Early stages of infection

DNA was detectable using nested-PCR within one hour of passage of infected blood and is likely to correlate directly to the number of parasites injected into the blood of the host animal and the high detection limit of the nested-PCR targeting the 18S rRNA reported by the assay used in this study (refer to Chapter five). Caution however should be taken in interpreting such early detection, as the intravenous passage of *B. gibsoni* is not comparable to the transfer of sporozoites by tick vectors or by possible direct blood-to-blood transmission of this parasite. Further research is therefore necessary to determine the detection limit of PCR during pre-acute stages of natural infections. Dogs could be experimentally infected with *B. gibsoni* using known infective ticks and parasite levels monitored from the initial time of tick attachment.

While IFAT gave a positive result on day one for each of the three dogs, this is most likely due to the transferral and detection of antibodies from the donor animal to the experimental dog and is further supported by the fall in titre during the early phase of infection in two of the dogs (A and C). The differences in antibody titres between dogs may be a consequence of Dog A and C receiving blood from chronically infected American Pit Bull Terriers, while Dog B received blood from Dog A during the acute phase of infection. The time taken to reach a titre of 1 : 160 was a more accurate indicator of the development of an immune response by each experimental dog and ranged from five days to two weeks. Previous studies have reported that host generated antibodies are first detected eight days (Fukomoto *et al*., 2001) or two weeks after inoculation with *B. gibsoni* (Anderson *et al*., 1980). Some dogs have also been reported not to seroconvert and IFAT would consequently fail to detect infection (Farwell *et al*., 1982). It is therefore important to consider using both PCR and IFAT to ensure the most accurate means of detecting *B. gibsoni* during all phases of infection.

ii) Chronic stages of infection

IFAT antibody titres were shown to be consistently greater than 1 : 640 during chronic stages of infection indicating a 100 % sensitivity of this test during the chronic experimental stages of experimental infection. However, the potential duration of elevated antibody levels is unknown. A study on *B. canis* infection showed a gradual decline in antibody titres and suggested the previously infected dogs did not remain in a state of premunition (Brandao *et al.*, 2003), while studies of *Plasmodium vivax* reported the potential persistence of specific antibodies against this parasite seven years after brief exposure (Braga, 1998). No study has definitively shown the total eradication of *B. gibsoni* once a dog has become infected, thereby making it difficult to determine whether there is a persistence of antibodies post-elimination of infection. Dogs in the study described in this chapter were unable to be kept alive for a longer period of time due to ethical considerations.

In contrast to IFAT, PCR detection was intermittent during some chronic stages of infection and reveals the potential for false negative results using this technique. The intermittent detection of *B. gibsoni* DNA using PCR in two of the experimentally infected dogs suggested that the level of infection was either below the detection limit of the PCR, or that parasites were only occasionally present within the venous blood system during these times. This study has shown that chronic infections are detected by PCR 43.6 to 100 % of the time. Other studies have however suggested that some dogs remaining consistently PCR positive during chronic infections, up to 220 days after the dog first became infected (Fukumoto *et al.*, 2001), further illustrating the variability of infection dynamics. *Babesia gibsoni* could also only be detected by secondary round PCR for 76 out of 78 days for one dog highlighting the importance of using nested PCR to increase the limit of detection (refer to Chapter five, section 5.4.1). To accurately detect piroplasm DNA using PCR during chronic stages of infection, it has been suggested that by testing at two or more time points, the diagnostic test sensitivity can be significantly increased (Calder *et al.*, 1996) and this requires further investigation regarding *B. gibsoni* infections.

8.5.4 Detection of B. gibsoni in tissues

This study also describes for the first time, a dog with a PCR negative blood result and a tissue positive result. Interestingly, the only tissue to be shown to be PCR positive was the lymph node. It is difficult to determine whether the presence of *B. gibsoni* DNA in the lymph node of this dog was from viable parasites or circulating degraded DNA present from the former infection. A recent study has suggested that DNA from non-viable *Plasmodium chaboudi* parasites is undetectable after 48 hr from the time when dead parasites were injected into the blood of mice (Jarra and Snounou, 1998). This suggests that PCR amplification of parasite DNA within blood is reflective of the presence of viable parasites and could also be assumed to be similar for piroplasm infections. Dead parasites are rapidly removed from the circulation by circulating and reticuloendothelial phagocytes and consequnetly causing the degradation of parasite DNA during phagocytosis (Jarra and Snounou, 1998).

The absence of circulating parasite within the venous blood but presence within lymphatic and splenic tissue may also relate to possible sequestration of *B. gibsoni* infected erythrocytes within tissues with high levels of capillary vasculature. Many *Plasmodium* spp. as well as *B. bovis*, *B. canis* and possibly Piroplasmida sp. (WA1) have been shown to exhibit sequestration through the process of cytoadhesion (Dao *et al.*, 1996; Schetters *et al.*, 1998; O'Connor *et al.*, 1999; Allred and Al-Khedery, 2004). Erythrocytes infected with parasite within the venous blood can then become sequestered in the capillaries of multiple host organs (O'Connor *et al.*, 1999; O'Connor and Allred, 2000). It is also reported that some species of piroplasm such as *B. bigemina* (O'Connor *et al.*, 1999), are non-sequestering, and further research into the possibility of sequestering is of *B. gibsoni* is therefore necessary.

The amplification of *B. gibsoni* DNA in the urine of one experimental dog, when no circulating parasite DNA was detected within the venous blood, was unexpected and may

have been the consequence of contamination occurring when the sample was collected by cystocentesis during necropsy. Degraded parasite DNA may also be filtered into the urine by the kidneys.

8.5.5 Conclusion

Overall, this study has suggested that combined atovaquone and azithromycin drug therapy can significantly lower *B. gibsoni* parasite levels, however totally eradication is unlikely and resistance to this drug therapy may develop. This study has also shown that PCR can be an effective tool in detecting early stages of infection, however can fail to accurately detect chronic and subclinical *B. gibsoni* infections due to the absence of circulating parasite in the venous blood. IFAT by contrast, may not effectively detect infection during the early stages but is very useful in the detection of *B. gibsoni* in chronic or carrier animals. It is therefore suggested that a combination of PCR and IFAT be used to increase the chances of accurately detecting *B. gibsoni*. Microscopy should only be considered useful for detecting acute stages of infection. This chapter has also described the detection of *B. gibsoni* in tissue samples using PCR for the first time and further research into tissue sequestration for this protozoan is necessary.

CHAPTER NINE

Canine Infectious Cyclic Thrombocytopenia in Australia

9.1 PCR-based investigation of the distribution and genetic variation of *A. platys* in Australia

9.1.1 Introduction

The absence of clinical signs in some dogs, the cyclic nature of CICT and low level parasitaemia have made diagnosis of this disease problematic. Microscopic examination of thin blood smears has limited use due to the difficulty in distinguishing platelet granules from *A. platys* morulae, combined with low sensitivity and specificity (Simpson and Gaunt 1991; Bradfield *et al.* 1996; Chang and Pan 1996; Inokuma *et al.* 2002). Reports suggest that IFA testing appears to be relatively species specific, however, fails to differentiate between current infection and previous exposure to *A. platys* (French and Harvey 1983; Chang and Pan 1996). Molecular based detection shows greater promise over microscopy and serological methods, exhibiting both high sensitivity and specificity (Chang and Pan 1996; Inokuma *et al.*, 2001c)**.**

Within Australia, *A. platys* was first detected in dogs of a remote community in the Tanami Desert, in central Northern Territory by PCR (Brown *et al.*, 2001). A subsequent study, investigating a clinical syndrome, often referred to as 'tick fever' that includes the clinical signs of depression, fatigue, fever, pale mucous membranes and bleeding tendencies, in pet dogs in northern Australia reported infections with *A. platys* (Jefferies, 2001). The majority of cases were found to be associated with thrombocytopenia and 27.8 % of these dogs were

infected with *A. platys*. This study further investigates *A. platys* infection in regions of Australia outside of the Northern Territory and the association of this infection with thrombocytopenia.

9.1.2 Aims

i. To investigate whether *A. platys* exists in the Australian states of Western Australia, Queensland, New South Wales and Victoria and assess the prevalence of this pathogen in thrombocytopenic dogs

ii. To genetically characterise isolates of *A. platys* from various geographical locations within Australia on the basis of the 16S rRNA gene and compare to other isolates worldwide

9.1.3 Materials and methods

i) Sample collection

Canine EDTA blood samples (n = 283) were collected from veterinary diagnostic pathology laboratories in Western Australia (Perth, Vetpath Laboratories), Queensland (Brisbane, IDEXX), New South Wales (Sydney, IDEXX) and Victoria (Melbourne, IDEXX) during the spring and summer months of 2003/04. Blood samples were categorized as either thrombocytopenic (platelets < 100 $x10^9$/L) or as non-thrombocytopenic (platelets >100 $x10^9$/L) (Table 9.1).

Location (State)	EDTA blood samples (Platelets <100)	(Platelets >100)	Total
WA	40	45	85
QLD	44	2	46
NSW	51	48	99
VIC	36	17	53
Total	**171**	**112**	**283**

Table 9.1

Canine blood samples collected from various Australian states (WA – Western Australia, QLD – Queensland, NSW – New South Wales, VIC – Victoria).

DNA was extracted from the EDTA blood samples according to Chapter four, section 4.2. A semi-nested set of primers was used for the amplification of a partial region of the 16S rRNA gene of *A. platys* (Table 9.2). The external primer (Ana R1) was designed using *A. platys* sequence information from the GenBank database (http://www.ncbi.nlm.nih.gov/entrez/, accessed 2/2003). The external primers (Ana R1 and PLATYS-F) produced a product size of 870 bp and the internal primers (PLATYS-F/R) produced a 504 bp product.

Primer name	Orientation	Sequence (5'-3')	Reference
Ana R1	Reverse	GCATCGAATTAAACCACATGC	This study
PLATYS-F	Forward	AAGTCGAACGGATTTTTGTC	Inokuma *et al.*, 2001
PLATYS-R	Reverse	CTTTAACTTACCGAACC	Inokuma *et al.*, 2001

Table 9.2

Primers used for the amplification of *A. platys* 16S rRNA gene

One µl of extracted DNA was added to a 24 µl reaction mixture comprising 0.6875 units of Tth Plus DNA polymerase (Fisher Biotech, Australia), 200 µM of each dNTP, 12.5 pmoles of the forward and reverse primers (Invitrogen, Australia), 2.5 µl 10x PCR buffer (Fisher Biotech, Australia) and 1.5 µl $MgCl_2$ (Fisher Biotech, Australia). Positive (1 µl of *A. platys*

DNA, Darwin, Australia) and negative (1 µl dH_2O) control samples were included with each set of PCR reactions.

Amplification was performed on a GeneAmp PCR system 2700 thermal cycler (Applied Biosystems, USA). For the primary round of amplification, an initial activation step at 94 C for 3 min, 62 C for 1 min and 72 C for 2 min, was followed by 45 cycles of amplification (94 C for 30 sec, 62 C for 20 sec and 72 C for 30 sec) and a final extension step of 72 C for 7 min for 25 µl reactions. The same conditions were followed for the secondary round of amplification, except that the annealing temperature was 55 C, using 1µl of DNA template from the primary reaction. Amplified DNA was electrophoresed and visualised according to Chapter four, section 4.4.

iv) Determination of specificity of PCR assay

Primer specificity was determined by using the BLAST 2.1 program (http://www.ncbi.nlm.nih.gov/BLAST/, accessed 2/2003). The specificity of the *A. platys* PCR was also determined by testing the assay against DNA of *Ehrlichia canis, Ehrlichia equi, Bartonella vinsonii, Rickettsia rickettsia* (kindly donated by Edward Breitschwerdt, North Carolina State University, USA) and canine DNA. *Anaplasma platys* DNA from Venezuela and Australia was used as positive control samples.

v) DNA sequencing and phylogenetic analysis

Amplified products were sequenced according to Chapter four, sections 4.5 – 4.8. Sequences were aligned, together with addition sequences from the GenBank database (Table 9.3) using Clustal W (Thompson *et al*., 1994).

Phylogenetic analysis was conducted on the basis of distance (Tajima and Nei, 1984) algorithms and tree topologies were inferred using Neighbour joining (Saitou and Nei, 1987)

using TREECON version 1.3b (Van de Peer and De Wachter, 1993). Statistical support for each tree was determined by using 1000 bootstrap replicates.

Species	Geographical orgin	Host	Accession No
A. platys	Okinawa, Japan	Dog	AF536828
A. platys	China	Dog	AF156784
A. platys	Thailand	Dog	AF286699
A. platys	Spain	Dog	AY530806
A. platys	Venezuela	Dog	AF399917
A. platys	Democratic Republic of Congo	Dog	AF478131
A. platys	Spain	Dog	AF303467
Anaplasma sp.	South Africa	Dog	AY570539
A. ovis	China	Sheep	AY262124
A. marginale			AF309867
Anaplasma sp.	California, USA	Llama	AF309867
A. centrale			AF318944
A. bovis		Cow	AY144729
Ehrlichia ewingii		Dog	M73227

Table 9.3

16S rRNA gene sequences for *A. platys* and related species obtained from the GenBank database (http://www.ncbi.nlm.nih.gov/entrez/).

9.1.4 Results

i) Determination of specificity of PCR assay

The PCR assay did not amplify DNA of *E. canis, E. equi, B. vinsonii* or *R. rickettsia* and also did not amplify host DNA for both primary and secondary reactions (Figure 9.1). Amplification was observed for each of the positive control *A. platys* samples from Australia and Venezuela.

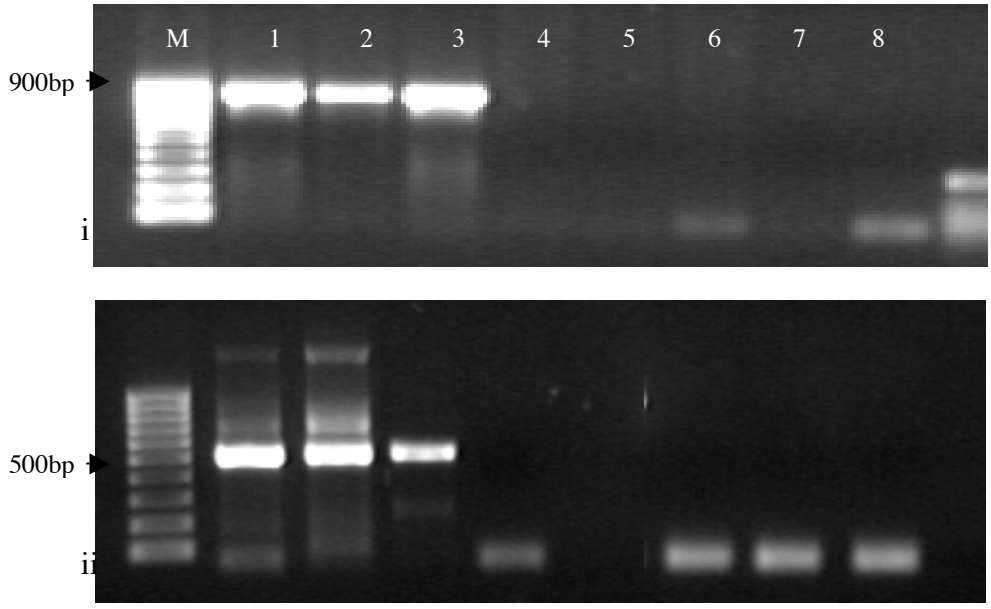


Figure 9.1

Specificity of primary (i) and secondary (ii) PCR reactions (Lanes 1-3 *Anaplasma platys*, 4 - *Ehrlichia canis*, 5 - *Ehrlichia equi*, 6 - *Bartonella vinsonii*, 7 - *Rickettsia rickettsia*, 8 – canine DNA).

ii) Amplification of A. platys DNA

Of the 283 samples screened by PCR, six were found to contain amplifiable *A. platys* DNA (Table 9.4). All six samples (3.5 %) were from thrombocytopenic dogs (n = 171).

Sample code	**Location**
WA1	Port Hedland, Western Australia
WA2	Broome, Western Australia
WA3	Perth, Western Australia
B1	Brisbane, Queensland
B2	Brisbane, Queensland
M1	Melbourne, Victoria

Table 9.4

Anaplasma platys positive blood samples from various locations within Australia.

iii) Genetic variation and phylogenetic analysis

DNA sequences from each of the six *A. platys* isolates were all 100 % homologous on the basis of a partial region of the 16S rRNA gene. Sequences were also identical to those obtained for isolates from the Northern Territory (refer to section 9.2). Australian sequences were compared to isolates from other geographical locations from around the world using phylogenetic analysis (Figure 9.2).

All *A. platys* isolates formed a single clade and were most closely related to *Anaplasma bovis* and an unnamed *Anaplasma* sp from a dog in South Africa. Two individual groups of *A. platys* was observed. The Australian isolates of *A. platys* clustered together with isolates from China, Japan, Thailand, Spain and France, while a second group contained isolates from Venezuela and The Democratic Republic of Congo. Statistic support for the separation of the formation of a single clade for all *A. platys* isolates was significant (83 %), however only moderate support was given for the separation of the *A. platys* isolates into two distinct groups (60 –61 %).

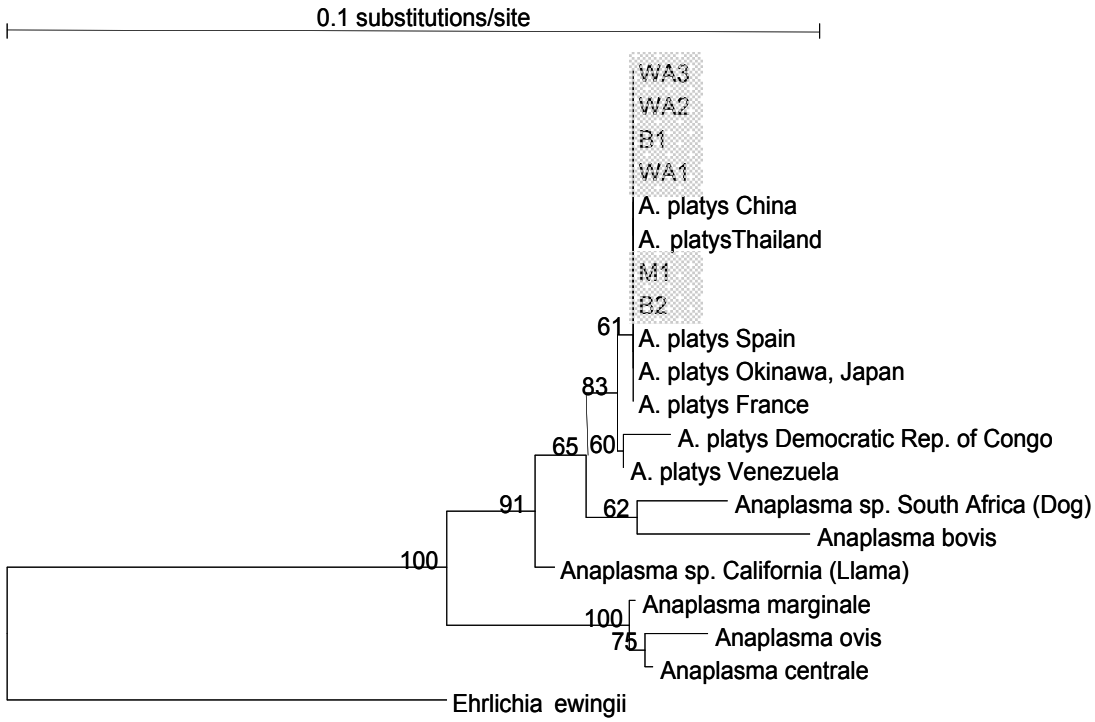


Figure 9.2

Phylogenetic tree constructed using a partial 16S rRNA gene sequences based on distance (Tajima Nei) and Neighbour joining analysis. Numbers above branches represent bootstrap percentages of 1000 replicates. Isolates from this study are shaded.

Four variable nucleotide sites were found to exist between the *A. platys* isolates from The Democratic Republic of Congo, Venezuela and all other isolates (Figure 9.3).

```
Venezuela                      TTTATCGCTATTAGATGAGCCTATGTTAGATTAGCTAGTTGGTAGGGTAA 150
Australia                      TTTATCGCTATTAGATGAGCCTATGTTAGATTAGCTAGTTGGTAGGGTAA 150
Democratic Republic of Congo   TTTATCGCTATTAGATGAGCCTATGTTAGATTAGCTAGTTGGTAGGGTAA 150
                               **************************************************

Venezuela                      AGGCCTACCAAGGCGGTGATCTATAGCTGGTCTGAGAGGATGATCAGCCA 200
Australia                      AGGCCTACCAAGGCAGTGATCTATAGCTGGTCTGAGAGGATGATCAGCCA 200
Democratic Republic of Congo   AGGCCTACCAAGGCGGTGATCTATAGCTGGTCTGAGAGGATGATCAGCCA 200
                               **********.*********************************** 

Venezuela                      CACTGGAACTGAGATACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGA 250
Australia                      CACTGGAACTGAGATACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGA 250
Democratic Republic of Congo   CACTGGAACTGAGATACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGA 250
                               **************************************************

Venezuela                      ATATTGGACAATGGGCGCAAGCCTGATCCAGCTATGCCGCGTGAGTGAGG 300
Australia                      ATATTGGACAATGGGCGCAAGCCTGATCCAGCTATGCCGCGTGAGTGAGG 300
Democratic Republic of Congo   ATATTGGACAATGGGCGCAAGCCTGATCCAGCTATGCCGCGTGAGTGAGG 300
                               **************************************************

Venezuela                      AAGGCCTTAGGGTTGTAAAACTCTTTCAGTGGGGAAGATAATGACGGTAC 350
Australia                      AAGGCCTTAGGGTTGTAAAACTCTTTCAGTGGGGAAGATAATGACGGTAC 350
Democratic Republic of Congo   AAGGCCTTAGGGTTGTAAAACTCTTTCAGTGGGGAAGATAATGACGGTAC 350
                               **************************************************

Venezuela                      CCACAGAAGAAGTCCCGGCAAACTCCGTGCCAGCAGCCGCGGTAATACGG 400
Australia                      CCACAGAAGAAGTCCCGGCAAACTCCGTGCCAGCAGCCGCGGTAATACGG 400
Democratic Republic of Congo   CCACAGAAGAAGTCCCGGCAAACTCCGTGCCAGCAGCCGCGGTAATACGG 400
                               **************************************************

Venezuela                      AGGGGGCAAGCGTTGTTCGGAATTATTGGGCGTAAAGGGCATGTAGGCGG 450
Australia                      AGGGGGCAAGCGTTGTTCGGAATTATTGGGCGTAAAGGGCATGTAGGCGG 450
Democratic Republic of Congo   AGGGGGCAAGCGTTGTTCGGAATTATTGGGCGTAAAGGGCATGTAGGCGG 450
                               **************************************************

Venezuela                      TTCGGTAAGTTAAAGGTGAAATGCCAGGGCTTAACCCTGGAGCTGCTTTT 500
Australia                      TTCGGTAAGTTAAAGGTGAAATGCCAGGGCTTAACCCTGGAGCTGCTTTT 500
Democratic Republic of Congo   TTCGGTAAGTTAAAGGTGAAATGCCAGGGCTTAACCCTGGAGCTGCTTTT 500
                               **************************************************

Venezuela                      AATACTGCCAGACTCGAGTCCGGGAGAGGATAGCGGAATTCCTAGTGTAG 550
Australia                      AATACTGCCAGACTCGAGTCCGGGAGAGGATAGCGGAATTCCTAGTGTAG 550
Democratic Republic of Congo   AATACTGCCAGACTCGAGTCCGGGAGAGGATAGCGGAATTCCTAGTGTAG 55
                               **************************************************

Venezuela                      AGGTGAAATTCGTAGATATTAGGAGGAACACCAGTGGCGAAGGCGGCTAT 600
Australia                      AGGTGAAATTCGTAGATATTAGGAGGAACACCAGTGGCGAAGGCGGCTAT 600
Democratic Republic of Congo   AGGTGAAATTCGTAGATATTAGGAGGAACACCAGTGGCGAAGGCGGCTAT 600
                               **************************************************

Venezuela                      CTGGTCCGGTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGAT 650
Australia                      CTGGTCCGGTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGAT 650
Democratic Republic of Congo   CTGGTCCGGTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGAT 650
                               **************************************************

Venezuela                      TAGATACCCTGGTAGTCCACGCTGTAAACGATGAGTGCTGA 691
Australia                      TAGATACCCTGGTAGTCCACGCTGTAAACGATGAGTGCTGA 691
Democratic Republic of Congo   TAGATACCCTGGTAGTCCACGCTGTAAACGATGA-TGCCTA 690
                               **********************************.***  *
```

Figure 9.3

Clustal W alignment of a partial region of the 16S rRNA gene of *A. platys* isolates from Australia, Venezuela and the Democratic Republic of Congo (Variable nucleotides are shaded).

9.1.5 Discussion

i) Distribution and prevalence

This study reports for the first time, the presence of *A. platys* in Western Australia, Queensland and Victoria. Unfortunately, samples were unavailable from South Australia and Tasmania, preventing assessment of infection of dogs within these States. Previously, *A. platys* has only been detected in dogs from the Northern Territory, in both central (Brown *et al.*, 2001; Brown *et al.*, 2005) and northern (Jefferies, 2001) geographical regions. It can now be assumed that *A. platys* is distributed throughout Australia and is likely to be a consequence of the extensive distribution of *R. sanguineus*, the suspected vector of this pathogen and unrestricted movement of dogs around the country.

Whilst *R. sanguineus* has not been successfully proven to transmit *A. platys* experimentally (Simpson *et al.*, 1991) DNA of *A. platys* has been detected using PCR within semi-engorged ticks (Sanogo *et al.*, 2003; Brown *et al.*, 2005). It is difficult to ascertain whether the *A. platys* DNA detected was simply a reflection of the ingestion of infected blood or whether *A. platys* was actually within the haemolymph of the tick. Limited study has assessed the distribution of *R. sanguineus* in Australia, however reports suggest that this tick species is prevalent throughout northern Western Australia, the Northern Territory, Queensland and northern New South Wales (Roberts, 1970). While large populations of this tick species are found within tropical to subtropical regions of Australia, there have also been reports in more temperate climatic regions in southern Australia including urban areas such as Perth (De Chaneet, 1976) and Melbourne (Roberts, 1970).

Brown *et al.* (2005) also postulated that the dog chewing louse (*Heterodoxus spiniger*) may be a potential vector of *A. platys* or may contribute to the spread of this pathogen by mechanical transmission. Transmission of *A. platys* is likely to be multifactorial, including the possibility of transplacental transmission (Brown *et al.*, 2005) and requires additional research to better understand the epidemiology of this organism.

The prevalence of *A. platys* infection among thrombocytopenic dogs sampled during the course of this pilot study was 3.5 % (6/171). This is much lower than the 27.8 % (5/18) prevalence observed in thrombocytopenic dogs in Darwin, Northern Territory (Jefferies, 2001). By selectively targeting a sub-sample of the normal population that presented with thrombocytopenia, it was anticipated that the likelihood of detecting *A. platys* would be increased. The prevalence of *A. platys* infection may therefore be much lower in the general dog population of Australia. This was reflected by the absence of infection in all non-thrombocytopenic blood samples tested, however the number of samples tested, especially from Queensland, were very small and not statistically significant. The results of this study however may not be a true reflection of the epidemiology of this disease in the general dog population of Australia, as while all dogs that were found to have *A. platys* were within the thrombocytopenic group, previous studies have suggested that many dogs infected with this pathogen do not present with thrombocytopenia and indeed may not show any signs of illness (Brown *et al.*, 2001; Jefferies, 2001; section 9.2). It is also difficult to definitively correlate the thrombocytopenia observed, with *A. platys* infection and may have been a consequence of an unrelated disorder such as immune mediated thrombocytopenia. A more accurate investigation into the prevalence of *A. platys* in dog populations in Australia therefore requires an increased sample size and sampling a much broader population, including obtaining detailed epidemiological and haematological data from each dog sampled.

Samples were also only collected from pathology laboratories of major Australian cities, disproportionately selecting samples from urban rather than rural dog populations. Dogs in rural areas could potentially have a higher rate of *A. platys* infection than their urban counterparts and should be investigated further.

ii) Genetic characterisation

This study has also revealed the existence of genetic differences between isolates of *A. platys* on the basis of the highly conserved, 16S rRNA gene and supports isolate variation reported by Mathew *et al.* (1997). The genetic variation observed between different isolates has not previously been correlated to geographic origin and the significance of this variation is not yet understood. The variability of clinical signs and pathogenesis of this infection sometimes observed in separate geographic regions of the world (Harrus *et al.*, 1997; Sainz *et al.*, 1999) may be explained by different genotypes and requires further investigation. No genetic variation in the 16S rRNA gene was observed between isolates from different regions within Australia and indeed, from multiple other countries. Characterisation on the basis of more variable gene loci is therefore required to better elucidate phylogeographical relationships among these isolates. Potential gene candidates include the citrate synthase gene (Raux *et al.*, 1997) and the major surface protein genes (de la Fuente *et al.*, 2002).

iii) Conclusion

This study has revealed that *A. platys* is likely to have a widespread distribution throughout Australia, extending beyond the Northern Territory and including Western Australia, Queensland and Victoria. All dogs with *A. platys* infection in this study were thrombocytopenic. Therefore, the possibility of *A. platys* infection should be considered by veterinarians, Australia-wide, when presented with cases of idiopathic thrombocytopenia. The prevalence of *A. platys* infection in Australia remains unknown and conformation of the vector responsible for the transmission of this organism is necessary and would facilitate future studies.

9.2 *Anaplasma platys* and *Babesia canis vogeli* infections in military German Shepherds from northern Australia

9.2.1 Introduction

Canine infections of *A. platys* are frequently associated with thrombocytopenia, which occurs in cycles of approximately 7-14 day intervals (Harrus *et al.*, 1997). Canine infectious cyclic thrombocytopenia is often reported to be subclinical, however this is disputed by other studies of infected dogs in southern Europe and the Middle East, which suggest that infection with *A. platys* results in weight loss, fever and depression (Harrus *et al.*, 1997; Sainz *et al.*, 1999). Such variable observations may be explained by differences attributable to the strain of the organism, the immune status of the host and by co-infection by one or more other organisms. No previous studies have investigated the clinical and pathological manifestations of *A. platys* infection in Australia.

Doxycycline has been suggested as an effective drug in the elimination of thrombocytopenia associated with *A. platys* infection (Bradfield *et al.*, 1996). Whether this drug therapy can produce total eradication of *A. platys* has not been proven and therefore requires additional investigation.

Anaplasma platys may also co-infect hosts with other pathogens such as *Ehrlichia*, *Babesia* and *Hepatozoon* spp. and has been reported in the USA, Thailand and (Kordick *et al.* 1999; Hua *et al.* 2000). Preliminary study suggested that co-infection of *A. platys* and *B. canis* does occur in northern Australia (Jefferies, 2001). Limited research however, has assessed the significance of co-infection and whether it produces any changes in the pathogenesis of these diseases.

9.2.1 Aims

- To investigate infections of *A. platys* and *B. canis* within clinically normal, military German Shepherds

- To assess the efficacy of doxycycline against *A. platys* infections

9.2.2 Materials and Methods

i) Dogs sampled

Blood samples were taken from fourteen German shepherd dogs in Darwin, Northern Australia, that were used for military purposes by the Royal Australian Airforce (RAAF). Blood was PCR screened for the presence of *Anaplasma platys* and for co-infection with *Babesia* species.

Eight of these dogs (six PCR positive for *A. platys*, two positive for *Babesia* and two not infected with *A. platys*) were involved in a further nine-week study. Blood was taken weekly and the packed cell volume (PCV), total white blood cells (WBC), platelet count and mean platelet volume (MPV) were calculated for each blood sample. Thin blood smears were also prepared for each sample and stained with a modified Giemsa Wright stain. Three dogs (all PCR positive for *A. platys* and one also co-infected with *Babesia*) were treated with doxycycline at 10 mg/kg once daily for 14 days. The weight, body temperature and food consumption of each of the dogs was recorded weekly and a score was given weekly for the dogs' ability and willingness to exercise through a standard agility training course (0 = unable to excise, 1-5 = reduced willingness to exercise, 5-8 = willingness to exercise and 8-10 = very willing and eager to exercise). A normal value was considered to be in the range of 5-10.

ii) PCR-screening for Anaplasma and Babesia

DNA was isolated from 200 µl aliquots of EDTA blood (stored at -20 C) using a QIAamp® DNA mini kit (QIAGEN, Hilden, Germany), according to Chapter four, section 4.2. The primers EHR16SD and EHR16SR (Table 9.5) were used to amplify an approximately 345 bp region of the 16S rRNA gene of most *Ehrlichia* and *Anaplasma* species. PIRO A1 (5' 3') and PIRO-B (5' 3') were used to amplify an approximately 450-bp region of the 18S rRNA gene of most *Babesia* species (Table 9.5). Sensitivity and specificity of this assay was calculated previously (Jefferies *et al.*, 2003).

Primer name	**Orientation**	**Sequence (5'-3')**	**Reference**
EHR16SD	Forward	GGTACCYACAGAAGAAGTCC	Parola, Roux *et al.* 2000)
EHR16SR	Reverse	TAGCACTCATCGTTTACAGC	(Parola, Roux *et al.* 2000)
PIRO A1	Forward	AGGGAGCCTGAGAGACGGCTACC	Jefferies *et al*, 2003
PIRO-B	Reverse	TTAAATACGAATGCCCCCAAC	Carret *et al*, 1999

Table 9.5

Primers used for the amplification of *A. platys* and *B. canis*

One µl of extracted DNA was added to a 24 µl reaction mixture comprising 0.625 units of HotStarTaq® DNA Polymerase (QIAGEN, Gernamy), 200 µM of each dNTP, 12.5 pmoles of each primer and 2.5 µl of 10 x PCR Buffer (containing 15 mM $MgCl_2$) (QIAGEN, Germany). Amplification was performed using a GeneAmp PCR System 2400 thermal cycler (Perkin Elmer, Foster City, California). For the *Ehrlichia*/*Anaplasma* PCR, an initial activation step at 95 C for 15 min, 55 C for 1 min and 72 C for 2 min was followed by 45 cycles of amplification (94 C for 30 sec, 55 C for 20 sec and 72 C for 30 sec) and a final

extension step of 7 min at 72 C. Amplification conditions for the *Babesia* PCR were the same except for an increased annealing temperature of 62 C.

iii) Sequencing of amplified products

The amplified products for both *A. platys* and *B. canis vogeli* were purified and sequenced according to Chapter four, sections 4.5 - 4.8.

iv) Statistical analyses

Statistical relationships between *A. platys* infection and selected clinical data were assessed using SPSS v11.0 (SPSS, Chicago, IL). Data was tested for normality using a test of homogeneity of variances before using a oneway ANOVA to test for significance between the presence of infection and clinical data. A Mann-Whitney test was also used to assess the relationship between *Babesia* infection and platelet number. A p-value of less than 0.05 was considered to indicate statistical significance.

9.2.3 Results

i) Presence of Anaplasma and Babesia DNA in blood samples

Six of the 14 dogs screened were PCR positive for *Anaplasma / Ehrlichia*. All blood samples were microscopy negative for platelet inclusions. Sequencing of the amplified product confirmed the presence of *A. platys* in each of the samples (100% homologous to *Anaplasma platys* strain Okinawa, GenBank accession number AF536828). Three of the dogs were PCR positive for *Babesia* and two were positive for both *Anaplasma* and *Babesia*. Sequencing of the amplified *Babesia* PCR product confirmed the presence of *Babesia canis vogeli* DNA in the samples (Details of the molecular characterisation of *B. canis* isolates from Australia are described in Chapter ten).

Blood samples from eight of the 14 dogs (six PCR positive for *A. platys*, two positive for *Babesia* and two infection free) showed variation in whether they were PCR positive for *A.*

platys and/or *B. canis vogeli* over the eight-week trial period. The presence or absence of infection with both haemoparasites is summarized in Figure 9.4.

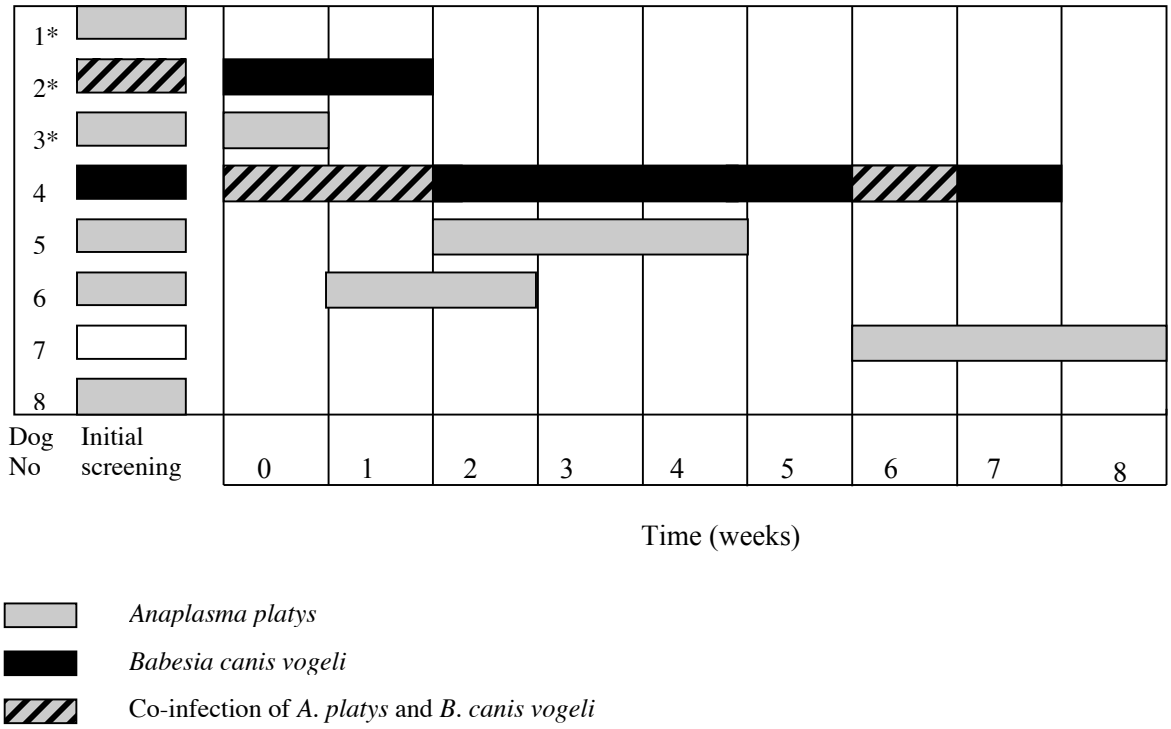


Figure 9.4

Infections of *Anaplasma platys* and *Babesia canis vogeli* over an eight-week trial period after an initial pre-trial screening (* denotes dogs which were treated with doxycycline for the first three weeks of the trial).

Over the entire period of blood collection, 100% of dogs were shown by PCR-screening to be infected with *A. platys*, 37.5% were infected with *Babesia canis vogeli* and 37.5% were infected with both parasites. None of the dogs were PCR positive for *A. platys* for more than three consecutive weeks.

Platelet number over the eight weeks for each of the eight dogs is shown in Figure 9.5. Dogs 3, 4, 5 and 8, each had platelet counts below 100 $x10^9$ /L. No significant correlation was found between the presence of *A. platys* and thrombocytopenia (platelets < 100) ($p = 0.456$). In addition, an infection with *A. platys* could not be correlated to PCV, total WBC, MPV, weight and temperature of each dog, food left by each dog or the exercise ability of each dog, when compared to values exhibited by dogs that were infection free. *Babesia* infection could also not be correlated to any clinical data or the dogs' weight, temperature and exerciseability.

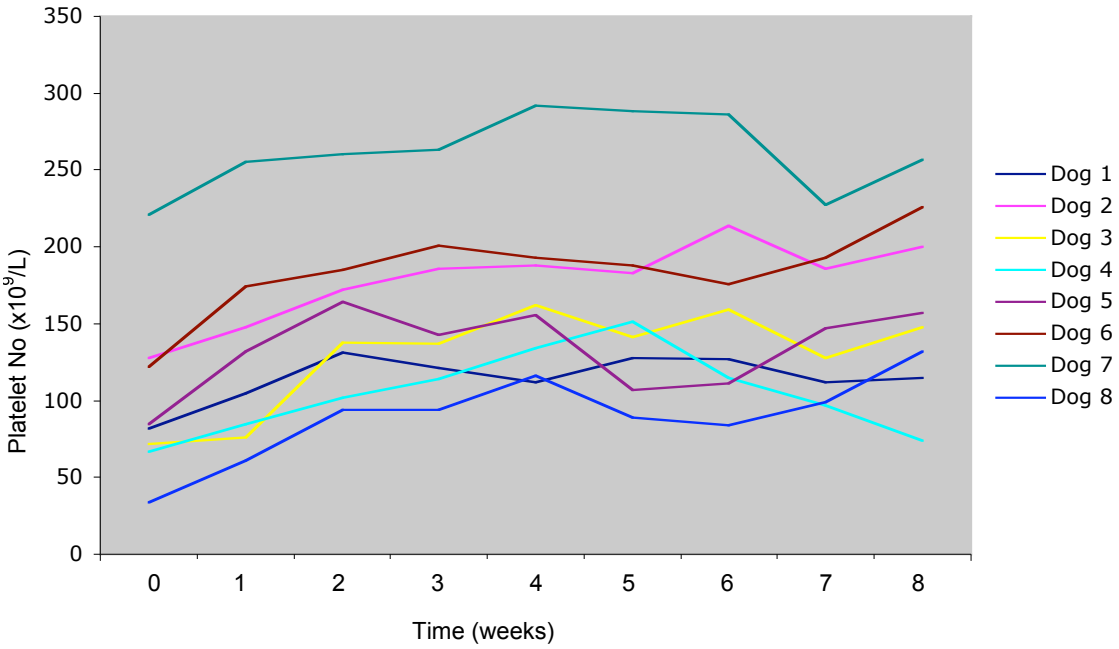


Figure 9.5

Plot of platelet number ($x10^9$/L) for each of the eight dogs studied over an eight week period

ii) Treatment with doxycycline

Each of the dogs that were treated with doxycycline was cleared of a detectable blood-borne *A. platys* infection. Doxycycline had no noticeable effect on *B. canis vogeli* infections.

9.2.3 Discussion

The results of this study suggest that chronic or repeat infections with *A. platys* in dogs in Australia are generally benign and that this organism may not be a significant pathogen. This concurs with previous studies in other parts of the world where detection of *A. platys* by PCR has not correlated with any major clinical signs (Chang *et al*., 1996; Brown *et al*., 2001; Inokuma *et al*., 2002). The intermittent detection of *A. platys* was suggestive of the cyclic nature of this infection and is in agreement with other studies that have reported cycles of infection occuring every 7– 14 days (Harrus *et al*., 1997). Daily sampling would be required to investigate the cyclic nature of the parasitaemia but has been previously studied in other countries (Chang and Pan, 1996; Chang *et al*., 1996; Chang *et al*., 1997).

Interestingly, the presence of *A. platys* DNA could not be correlated with thrombocytopenia. This may be a reflection of the low sample size influencing the statistical significance, inaccurate platelet counts or possibly related to the chronic stage of these infections. Furthermore, as blood samples were only collected once a week and not daily, it is possible that blood was not taken during periods of thrombocytopenia.

It still remains unknown, however, whether dogs in Australia exposed to *A. platys* for the first time produce clinical signs of increased severity. It could be speculated that the dogs within this study had been exposed regularly to infected ticks and may have developed some degree of immunity, hence the absence of ill health. Only by using experimental infections would it be possible to determine the pathogenesis of *A. platys* in previously naïve dogs in Australia.

Other studies have suggested the existence of different strains of *A. platys,* with one producing no detectable clinical signs and the other causing anorexia, depression, lethargy, lymphadenomegaly and fever (Harrus *et al*., 1997). It is possible that the difference in clinical signs may also be due to the stage of infection, age, breed, the immune status of the animal or the genotype of *A. platys*.

Treatment with doxycycline appeared to reduce the infection to an undetectable level after a week, however some untreated dogs also developed undetectable DNA levels of *A. platys.* It is therefore difficult to determine whether these results are a reflection of the cyclic nature of this disease or an effect of doxycycline. It remains inconclusive whether doxycycline is an effective treatment for *A. platys* infection in Australia. One previous study has suggested that tetracycline is a useful therapy for *A. platys* infection, however, the duration of treatment is dependent upon the stage of illness, with chronic infections requiring a longer period of drug therapy (Chang and Pan, 1997). Doxycycline has also been reported to be a more effective drug therapy than tetracycline (Chang *et al*., 1997).

This study is also the first known report of co-infection with *A. platys* and *B. canis vogeli* in dogs in Australia. Both parasites have been reported to be transmitted by the tick *R. sanguineus* (*A. platys* not experimentally confirmed), which is widely distributed across northern Australia. *Babesia canis vogeli* in Australia has previously been reported to be mildly pathogenic, causing severe anaemia and sometimes death in young dogs but may exist in a state of premunity or carrier state in adult dogs (Irwin and Hutchinson, 1991). Dogs infected with both *A. platys* and *B. canis vogeli* showed no detectable pathogenesis, however this may again be a reflection of the immune status of the animals. Co-infection in naïve animals may give rise to detectable clinical signs and pathogenesis and therefore requires further investigation.

Overall, the results from this study suggest that *A. platys* infection may be quite prevalent in dogs in northern Australia, however chronic or repeat infections are unlikely to cause any major symptoms. Regular tick prevention treatment should be considered as the most effective method of controlling infections of both *A. platys* and *B. canis vogeli.*

CHAPTER TEN

Molecular characterisation of the Australian canine *Babesia* spp. and phylogeographical relationships among worldwide isolates of *B. canis* and *B. gibsoni*

10.1 Introduction

A total of six individual species of piroplasm have been reported to infect dogs, these being *B. canis*, *B. gibsoni*, *Babesia* sp. (North Carolina), *B. conradae, T. annae* and *T. equi*. The species, *B. canis* is comprised of four subspecies; three of which; *B. canis canis, B. canis vogeli* and *B. canis rossi,* are each considered to be separate species by some (Uilenberg *et al*., 1989; Zahler *et al*., 1998; Carret *et al*., 1999), yet have never been taxonomically elevated. The fourth subspecies, *B. canis presentii*, has only been reported in cats (Baneth *et al*., 2004). Limited study has investigated the concept of the species within the canine piroplasms and the levels of inter- and intra-species genetic variation that exist within established species.

Multiple isolates of canine piroplasm have been defined as new species on the basis of molecular characterisation (Kjemtrup *et al*., 1999; Zahler *et al*., 2000; Birkenheuer *et al*., 2004), yet the level of genetic variation used to define a species has not been established. Defining levels of inter-species variation would thus limit the current difficulty and confusion that exists when describing a new species or genotype. Defining levels of intra-species genetic variation within individual species of piroplasmid is also important and is imperative to many fields of research, including diagnosis, epidemiology, chemotherapy, systematics and taxonomy. PCR is increasingly becoming a widely used detection technique

and accurate amplification of DNA of the target species is paramount. The existence of genetic variation may also be useful in determining the geographic origin of an isolate, phylogeographical relationships and also explaining possible variations in pathogenesis and life cycle characteristics.

Within Australia, both *B. canis* (Chapter six and nine) and *B. gibsoni* (Chapters six and seven) have been reported and provided the basis for the molecular characterisation and phylogeography described in this chapter. Isolates of both species were collected from different geographical locations within Australia and from around the world, and were used to investigate molecular variation, phylogeny and taxonomy.

10.2 Aims

- To collect isolates of canine piroplasms from various geographical locations worldwide.

- To molecularly characterise *B. gibsoni* and *B. canis vogeli* isolates from dogs in Australia on the basis of 18S rRNA gene, the ITS 1, 5.8S rRNA and ITS 2 loci and HSP 70 gene.

- To investigate levels of intra-species genetic variation using the 18S rRNA gene and ITS 1, 5.8S rRNA, ITS 2 loci and HSP 70 gene, among world-wide isolates of *B. gibsoni* and *B. canis* and determine levels of inter-species genetic variation among the canine piroplasms and other established species of the Piroplasmida

- To review the taxonomic status of the *B. canis* subspecies

10.3 Materials and Methods

10.3.1 Isolates collected

Whole canine blood samples (n = 30) and blood applied to FTA cards (n = 16), each known to be infected with piroplasm spp. by microscopic visualisation, were collected from various countries worldwide (Table 10.1).

Isolate code	Piroplasm size	Geographic origin	Acknowledgement
A1	Large	Queensland, Australia	Jefferies *et al.*, 2003
A2, A3	Large	Darwin, NT, Australia	This study (refer to Chapter nine)
A4	Large	Alice Springs, Australia	This study (refer to Chapter six)
A5	Small	Victoria, Australia	This study (refer to Chapter seven)
A6	Small	NSW, Australia	This study (refer to Chapter six)
M1*, M2	Large	Petaling Jaya,, Malaysia	Yeoh Eng Cheong, Yeoh Veterinary Clinic, Malaysia
M3*	Small	Malaysia	Yeoh Eng Cheong, Yeoh Veterinary Clinic, Malaysia
Th1*	Large	Thailand	Clare McKay and Rebecca Traub, Murdoch University
S1	Small	Singapore	John Jardine, Vetpath Laboratories, Western Australia
P1*	Large	Philippines	Roberto Puentespina, Animal Solutions Veterinary Hospital, Davao City, Philippines
T1	Small	Taiwan	John Jardine, Vetpath Laboratories, Western Australia
HK1-3*	Small	Hong Kong	Michael Goodlet, Stanley Veterinary Centre, Hong Kong; Brad Easton, Aberdeen Vet Clinic, Hong Kong
SL1-4*	Small	Sri Lanka	Nalinika Obeyesekere, Pet Vet Clinic, Colombo, Sri Lanka
B1-6	Large	Sao Paula , Brazil	Lucia O'Dwyer, Universidade Estadual Paulista, Brazil
B7-10	Small	Botucatu, Brazil	Cynthia Lucidi, Universidade Estadual Paulista, Brazil
U1*	Large	Montevideo, Uruguay	Graciela Oliver, Universidad de la Republica Uruguay, Montevideo, Uruguay
I1	Large	Nahariya, northern Israel	Gad Baneth, Hebrew University of Jerusalem, Israel
I 2, I3	Large	Central Israel	Gad Baneth, Hebrew University of Jerusalem, Israel
I 4	Large	Beer Sheva, Southern Israel	Gad Baneth, Hebrew University of Jerusalem, Israel
SA 1-5	Large	South Africa	Linda Jacobson, University of Pretoria, Ondesterpoort, South Africa
Sp 1	Large	Teneriffe Island, Spain	Monika Zahler, Institut fur vergleichende Tropenmedizin und Parasitologie, Germany
H 1-4*	Large	Hungary	Akos Mathe, Szent Istvan University, Budapest, Hungary
F 1	Large	France	Peter Irwin, Murdoch University
NC 1	Small	North Carolina, USA	Ed Breitschwerdt, North Carolina State University, USA

Table 10.1

Isolates of canine Piroplasmida spp. collected from various geographical locations worldwide (* denotes samples obtained using FTA cards).

10.3.2 DNA extraction

DNA was extracted from whole blood according to the protocol described in Chapter four, section 4.2. Discs (1.2 mm) were punched from the dried blood applied to FTA cards and purified according to Chapter five, section 5.3.7 and used for subsequent DNA amplification.

10.3.3 Amplification and sequencing of the 18S rRNA, ITS 1, 5.8S rRNA, ITS 2 and HSP 70 loci

Two sets of primers were used to amplify a partial region of the 18S rRNA gene using a nested assay and/or the complete 18S rRNA gene (Table 10.2). A semi-nested PCR assay was developed for the amplification of the entire ITS 1, 5.8S rRNA gene and ITS 2 by modifying procedures described by Zahler *et al*. (1998) and Holman *et al*. (2003) (Table 10.2).

Locus	Primer name	Orientation	Sequence (5' – 3')	Reference
18S rRNA complete	BT1-F	Forward	GGTTGATCCTGCCAGTAGT	Criado-Fornelio *et al*., 2003a
	BT2-R	Reverse	CTTCTGCAGGTTCACCTACG	
18S rRNA	BTF1	Forward	GGCTCATTACAACAGTTATAG	This study (Chapter five)
Partial	BTR1	Reverse	GAGAGAAATCAAAGTCTTTGGG	This study (Chapter five)
	BTF2	Forward	CCGTGCTAATTGTAGGGCTAATAC	This study (Chapter five)
	BTR2	Reverse	CGATCAGATACCGTCGTAGTCC	This study (Chapter five)
ITS 1, 5.8S rRNA, ITS 2	RIB-13	Forward	CCGAATTCTTTGTGAACCTTATCA	Zahler *et al*., 1998
	RIB-3	Reverse	CGGGATCCTTCRCTCGCCGYTACT	Zahler *et al*., 1998
	ITS F	Forward	GAGAAGTCGTAACAAGGTTTCCG	Holman *et al*., 2003
HSP 70	BGHsp70-F3	Forward	TCAAGGACTTCTTCAACGGA	Yamasaki *et al*., 2002
	BGHsp70-R	Reverse	CWTGTGHTTAGTCAACYTCCTCWAC	Yamasaki *et al*., 2002

Table 10.2

Primers used for the amplification of the 18S rRNA gene, ITS 1, 5.8S rRNA gene and ITS 2 of various canine piroplasm isolates

For each PCR reaction, 1 µl of extracted DNA or a purified 1.2 mm FTA disc was added to a 24 µl reaction mixture comprised of 0.6875 units of *Tth Plus* DNA polymerase (Fisher Biotech, Australia), 200 µM of each dNTP (Fisher Biotech, Australia), 12.5 pmoles of each primer, 2.5 µl of 10x PCR buffer (Fisher Biotech, Australia) and 1.5 µl of $MgCL_2$. Amplification was performed using a GeneAmp PCR thermal cycler (Perkin Elmer, California, USA). Cycling conditions for BTF1/R1 and BTF2/R2 are described in Chapter five, section 5.3.3. Cycling conditions for each of the other primer sets were identical except for different annealing temperatures, which were 58 C for BT-F/BT2-R, 60 C for RIB 3/RIB 13, 65 C for RIB 3/ITS F and 60 C for BGHsp70 F3/R.

Amplified DNA was purified and sequenced according to the protocol described in Chapter four, section 4.5 – 4.8.

10.3.4 Sequence alignment and phylogenetic analysis

Sequences obtained for each gene, in addition to sequences for the 18S rRNA gene (Table 10.3), the ITS1, 5.8S rRNA gene and ITS2 (Table 10.4) and HSP 70 gene (Table 10.5) obtained from the GenBank database (http://www.ncbi.nlm.nih.gov/entrez/) were aligned using Clustal W (Thompson *et al.*, 1994). A partial 18S rRNA gene sequence of *Babesia kiwiensis* (Down, 2004) was also included for phylogenetic analysis. Phylogenetic analysis was conducted on the basis of distance (Tajima and Nei, 1984) algorithms and tree topologies were inferred using Neighbour joining (Saitou and Nei, 1987) using TREECON version 1.3b (Van de Peer and De Watcher, 1993). Statistical support for each tree was determined by using 1000 bootstrap replicates. Percentage identity between isolates and species was calculated based on Kimura 2-parameter distance method using MEGA v.3 (Kumar *et al.*, 2004).

Species (host, geographical origin)	Accession No	Species (host, geographical origin)	Accession No
*B. canis vogeli (*Dog, Japan)	AB083374	*B. gibsoni, (Dog, Oklahoma,USA)*	AF205636
B. canis vogeli (Dog, Brazil)	AY371196, 95,94	*B. gibsoni* (Dog, Aomori, Japan)	AB118032
B. canis vogeli (Dog, USA)	AY371198	*B. gibsoni* (Dog, Spain)	AY278443
B. canis vogeli (Dog, Egypt)	AY371197	*B. gibsoni* (Dog, Georgia, USA)	AF396748, 49
B. canis vogeli (Dog France)	AY0729225	*B. gibsoni* (Dog, Okinawa, Japan)	AF271082
B. canis vogeli (Dog, Spain)	AY150061	*B. gibsoni* (Dog, Nth Carolina, USA)	AF271081
B. canis canis (Dog, Croatia)	AY072926	*B. gibsoni* Asia 1 (Dog, Japan)	AF175300
B. canis canis (Dog, Russia)	AY962186, 87	*B. gibsoni* Asia 2 (Dog, Malaysia, Sri Lanka)	AF175301
B. canis canis (Dog, Warsaw)	AY321119	*Babesia* sp. (Red cheeked souslik, Xinjing, China)	AB083376
B. canis canis (Dog, Slovakia)	AY780888	*Babesia* sp. Akita	AY190123
B. canis canis (Dog, Slovenia)	AY259123, 24	*Babesia* sp. (Bandicoot rat, Thailand)	AB053216
B. canis canis (Dog, Netherlands)	AY703070, 71,72,73	*B. odocoilei*	AY237638
B. canis rossi, (Dog, South Africa)	L19079	*Babesia* sp. RD1 (Reindeer)	AF158711
B. canis rossi (Dog, Sudan)	DQ111760	*Babesia* sp MO1 (Human, Missouri)	
B. canis presentii, (cat Israel)	AY272047	*Babesia* sp EU1 (Human)	AY046575
		B. divergens	U16370

Table 10.3

Additional 18S rRNA gene sequences of canine piroplasms and related species obtained from the GenBank database.

Species	Host	Geographical origin	Accession No
B. canis rossi	Dog	South Africa	AF394535
B. canis presentii	Cat	Israel	AY272048
B. caballi	Horse	Namibia	AF394536

Table 10.4

Additional ITS 1, 5.8S rRNA and ITS 2 sequences obtained from the GenBank database.

Species	Host	Geographical origin	Accession No
B. gibsoni	Dog	Korea	AB083512
B. gibsoni	Dog	Japan	AB083510
B. bovis	Cattle	Unknown	AF107118
T. annulata	Cattle	Unknown	J04653

Table 10.5

Additional HSP 70 gene sequences obtained from the GenBank database.

10.4 Results

10.4.1 Amplification and sequencing of the 18S rRNA gene

For all isolates (n = 43), except for the small piroplasm samples B6-9 from Brazil, a partial region of the 18S rRNA gene (850 bp) was amplified and sequenced. The complete 18S rRNA gene could not be amplified for all isolates. For all FTA card samples (n = 16), no product or a non-specific product of the expected product size was amplified (Figure 10.1). A non-specific product was also amplified for samples B6-9. Sequencing revealed the amplification of either mammalian or fungal DNA in these samples. Only partial 18S rRNA gene sequences were therefore used for phylogenetic analysis.

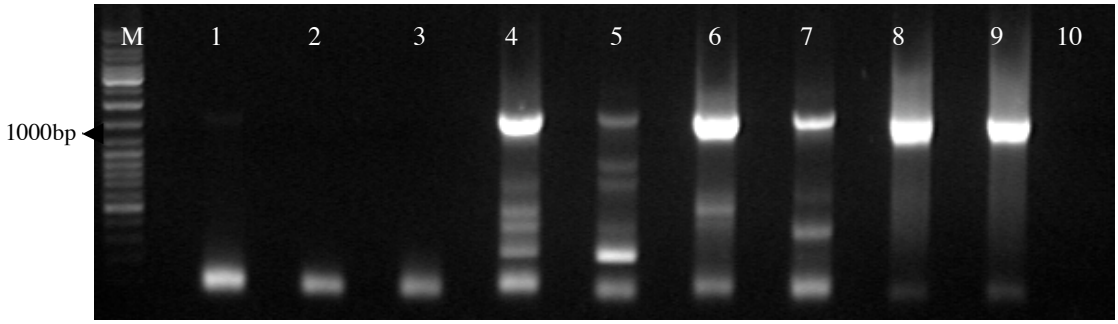


Figure 10.1

Amplification of the complete 18S rRNA gene (M – molecular marker, 1 – HK1, 2 – HK2, 3 – HK3, 4 – SL1, 5 – SL2, 6 – SL3, 7 – SL4, 8 – M1, 9 – U1, 10 – negative control)

10.4.2 Phylogeographical analysis using the 18S rRNA

On the basis of the partial 18S rRNA gene, all large piroplasm isolates were genetically most homologous to either *B. canis vogeli*, *B. canis canis* or *B. canis rossi* (Figure 10.2).

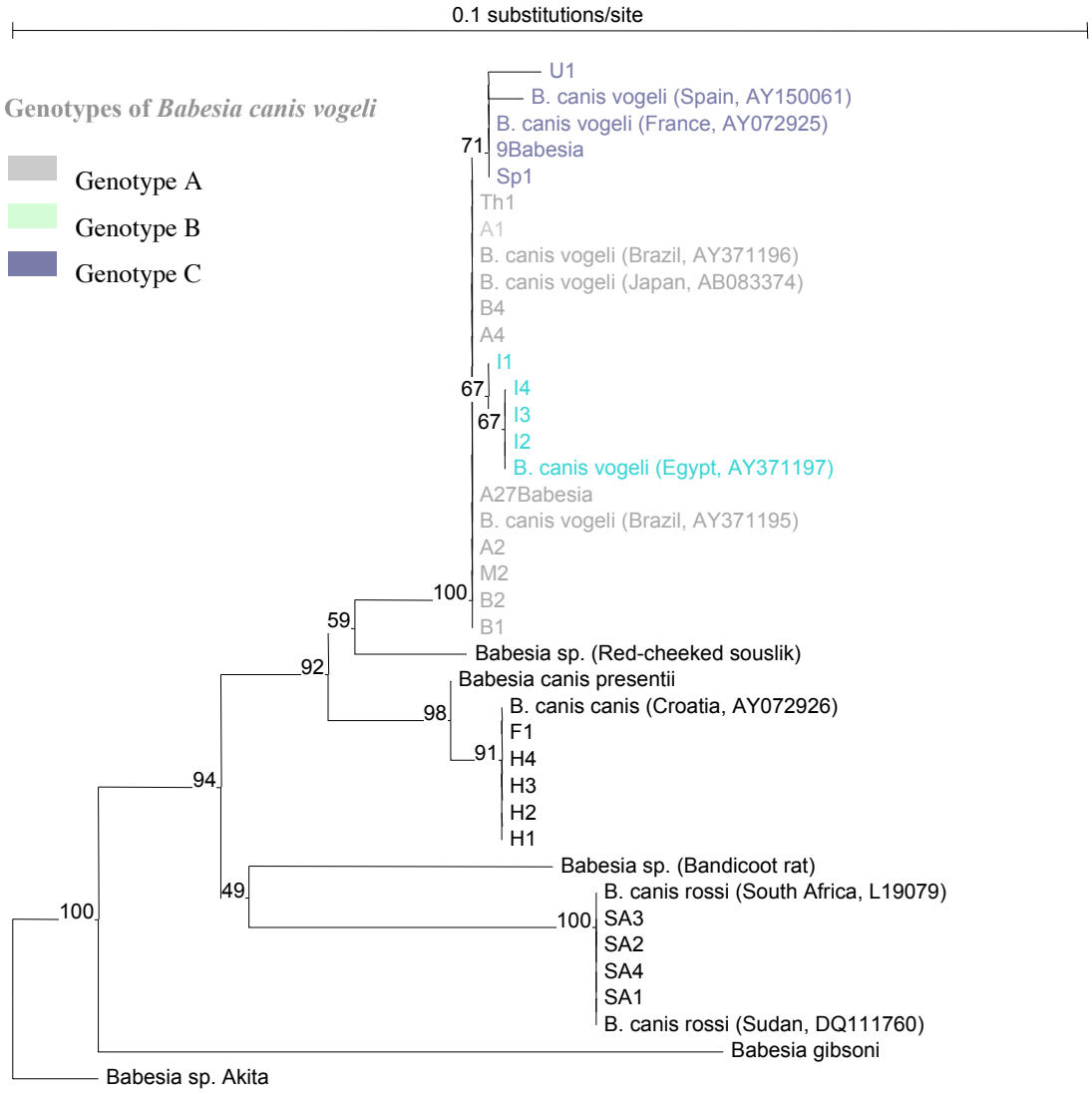


Figure 10.2

Phylogenetic tree constructed using partial 18S rRNA gene sequences based on distance (Tajima Nei) and Neighbour joining analysis. Numbers above branches represent bootstrap percentages of 1000 replicates. Coloured isolates represent possible genotype groups.

Variation was observed between isolates of *B. canis vogeli*, which formed three genogroups. Genotype A represents isolates from Australasia and Brazil, Genotype B includes isolates from Israel and Egypt and Genotype C represents isolates from Europe, Uruguay and the USA. Significant statistical support was provided for genotypes A (100 %) and C (71 %) and moderate support was given to genotype B (67 %).

The average homology observed between isolates of *B. canis vogeli* was 99.8% (Table 10.6) with the greatest level of variation observed between isolates from Israel (I1 - I4). No variation was observed between isolates of either *B. canis canis* or *B. canis rossi* on the basis of the partial 18S rRNA gene used for analysis.

	Bcr	**Bcc**	**Bcp**	**Bcv**	**BspRs**	**BspB**	**B.gib**
Bcr	100						
Bcc	94.5	100					
Bcp	95.1	99.5	-				
Bcv	94.9	97.3	97.9	99.8			
BspRs	95.1	97.7	97.8	98.0	-		
BspB	94.4	95.1	95.1	94.8	94.7	-	
B.gib	93.5	95.3	95.3	95.1	94.9	95.1	-

Table 10.6

Average percentage similarity of the 18S rRNA gene among and between species/subspecies using Kimura 2-parameter distance method (MEGA). Bcr – *B. canis rossi*, Bcc – *B. canis canis*, Bcp – *B. canis presentii*, Bcv – *B. canis vogeli*, BspRS – *Babesia* sp. Red-cheeked souslik, BspB – *Babesia* sp. Bandicoot rat, B.gib – *B. gibsoni*.

Percentage identity was also calculated between each of the *B. canis* subspecies and related species (Table 10.6). Variation within the species *B. canis* ranged from 99.5 % (between *B. canis canis* and *B. canis presentii*) to 94.5 % (Between *B. canis canis* and *B. canis vogeli*). A similar level of identity was observed between the *B. canis* subspecies and the *Babesia* sp.

from a Red-cheeked souslik (95.1 – 98 %), the *Babesia* sp. from a Bandicoot rat (94.4 – 95.1 %) and *B. gibsoni* (93.5 –95.1 %).

Analysis of a smaller region of the 18S rRNA gene (356 bp) which contained both variable and non-variable regions, allowed for the inclusion of additional sequences of *B. canis canis* obtained from the GenBank database (Figure 10.3).

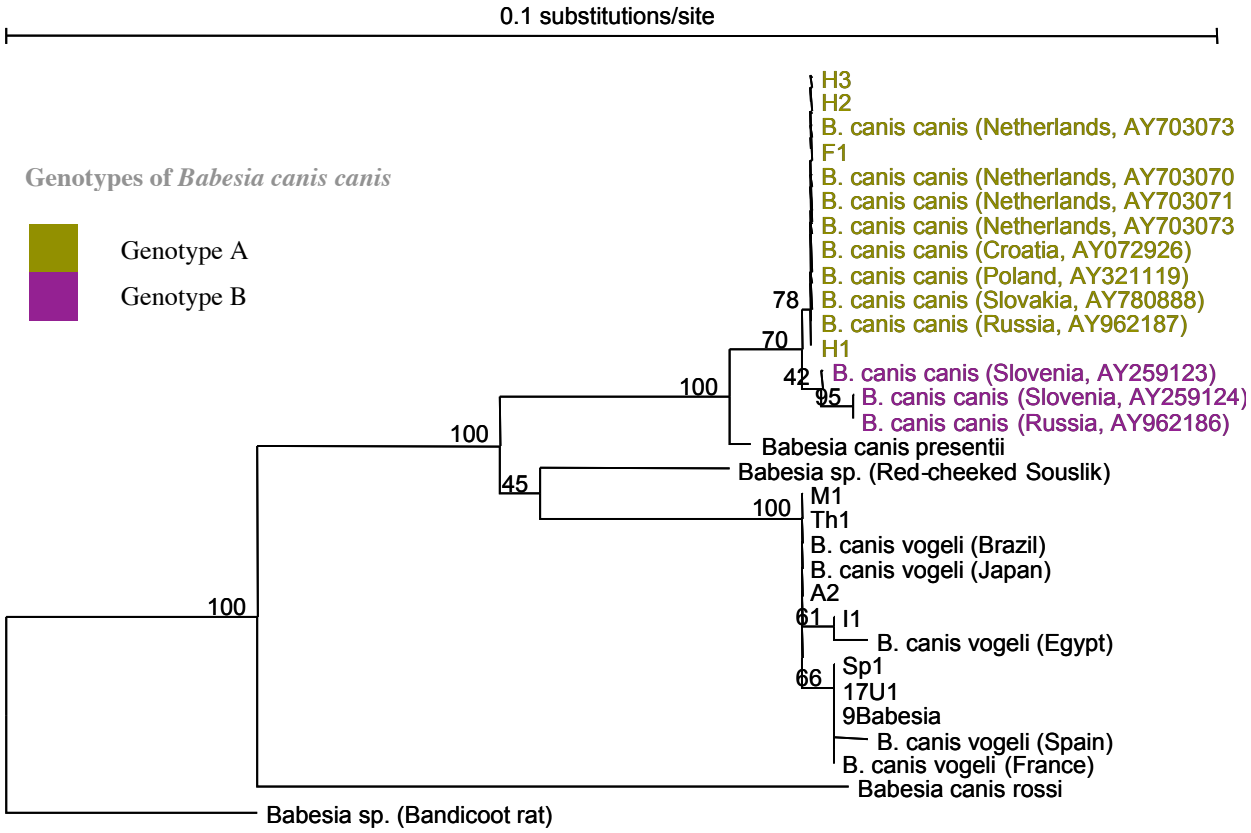


Figure 10.3

Phylogenetic tree constructed using a partial 18S rRNA gene sequences based on distance (Tajima Nei) and Neighbour joining analysis. Numbers above branches represent bootstrap percentages of 1000 replicates.

As with the subspecies *B. canis vogeli*, genogroups were also observed within *B. canis canis*. Genotype A represents isolates from Hungary, France, The Netherlands, Croatia, Poland,

Slovakia and Russia. Genotype B included isolates from Slovenia and Russia. Significant bootstrap support was given to Genotype A (78 %).

Further analysis using the partial 18S rRNA gene was conducted with the inclusion of *B. kiwiensis* (Figure 10.4). *Babesia canis canis, B. canis presentii*, *Babesia* sp. from a red-cheeked souslik and *B. canis vogeli* formed a clade together with strong bootstrap support, however the phylogenetic position of *B. kiwiensis* remained inconclusive. *Babesia canis rossi* and *Babesia* sp. from a bandicoot rat were the most ancestral species within this group.

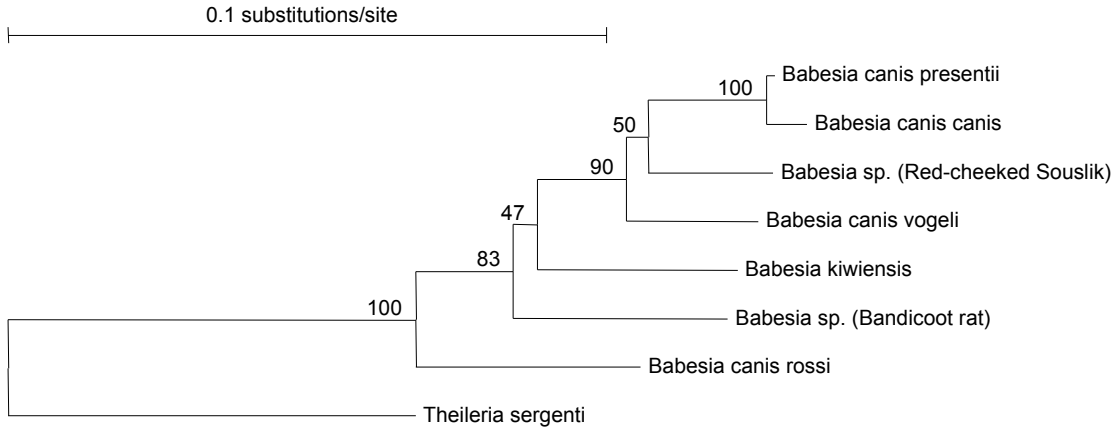


Figure 10.4

Phylogenetic analysis of the *B canis* subspecies with the inclusion of *B. kiwiensis* using a partial 18S rRNA gene sequences based on distance (Tajima Nei) and Neighbour joining analysis. Numbers above branches represent bootstrap percentages of 1000 replicates.

Levels of variation on the basis of the 356 bp partial region of the 18S rRNA are shown in Table 10.7. Identity between subspecies ranged from 99.1 –91.4 % and between species, ranged from 96.3 – 91.4%.

	Bcc	Bcp	Bsp. RS	Bcv	Bk	Bsp. B	Bcr
Bcc							
Bcp	99.1						
Bsp. RS	95.8	96.0					
Bcv	94.8	95.7	96.3				
Bk	92.3	92.3	92.9	93.9			
Bsp B	92.6	92.6	92.3	93.4	93.5		
Bcr	91.4	92.3	92.9	91.8	91.4	91.7	

Table 10.7

Average percentage similarity of the partial 18S rRNA gene between species/subspecies using Kimura 2-parameter distance method (MEGA). Bcr – *B. canis rossi*, Bcc – *B. canis canis*, Bcp – *B. canis presentii*, Bcv – *B. canis vogeli*, BspRS – *Babesia* sp. Red-cheeked Souslik, Bk – *B. kiwiensis*, BspB – *Babesia* sp. Bandicoot rat.

All isolates of small piroplasm clustered together with the species *B. gibsoni* on the basis of the 18S rRNA gene (Figure 10.5). Two distinct genotypes were observed with strong statistical support. Genotype A includes isolates from Australasia and the USA, while Genotype B is represented by a single isolate from Spain. Average identity between isolates of *B. gibsoni* was 99.4%. The most homologous species to *B. gibsoni* were *Babesia* sp. Fukui (95.5 %), *Babesia* sp. Akita (95.5 %) and *B. odocoilei* (94.9%).

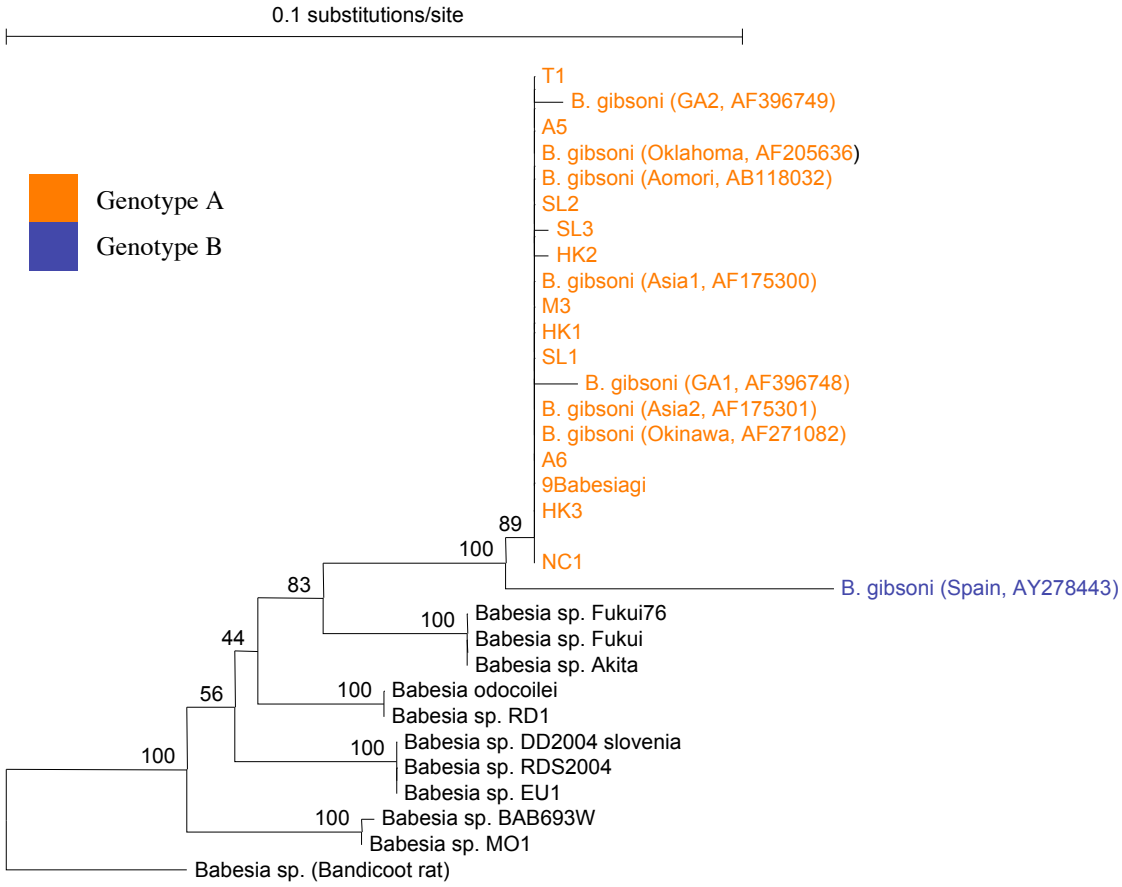


Figure 10.5

Phylogenetic analysis of *B. gibsoni* isolates and related species, constructed using a partial 18S rRNA gene sequences based on distance (Tajima Nei) and Neighbour joining analysis. Numbers above branches represent bootstrap percentages of 1000 replicates.

10.4.3 Amplification and sequencing of the ITS 1, 5.8S rRNA gene and ITS 2

The complete ITS 1, 5.8S rRNA gene and ITS 2 (approximately 800bp) were amplified for the large isolates B2-5, M2, A2, I2, H1, F1, SA1 and SA2 and for the small isolates A5 and NC1. All other isolates (n = 33) were either unable to be amplified or were not sequenced due to time and resource limitations. Distance-based phylogenetic analysis showed the separation of each subspecies/species with 100 % bootstrap support (Figure 10.6).

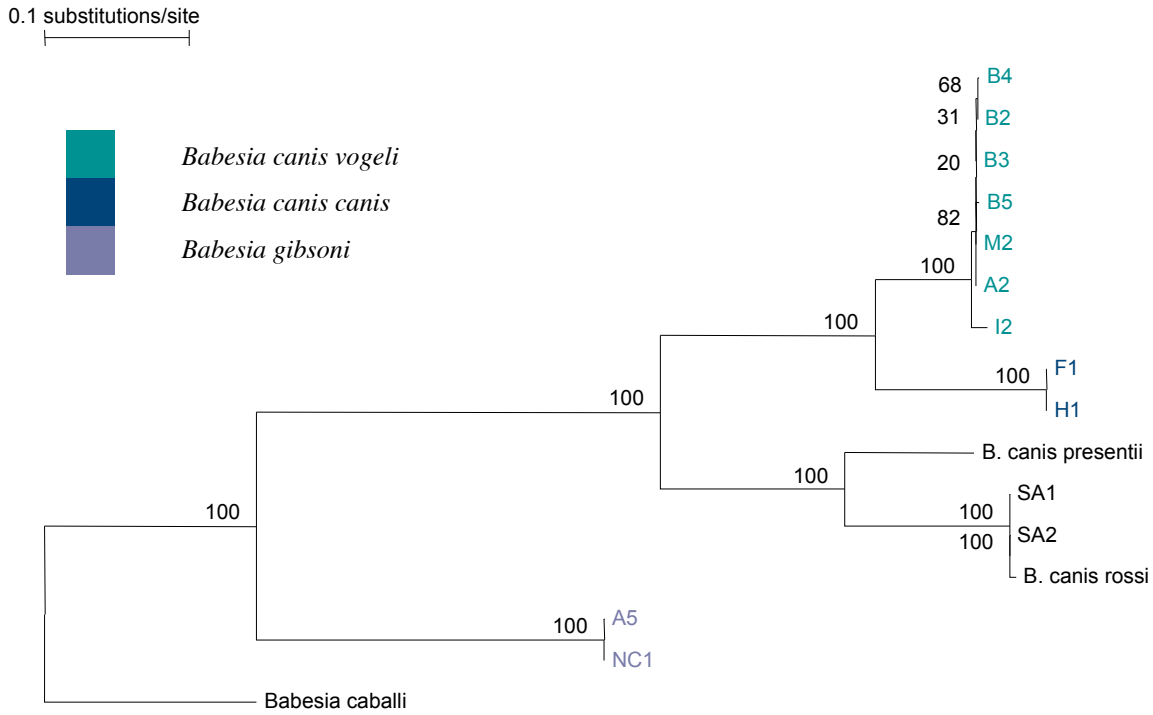


Figure 10.6

Phylogenetic tree constructed using the ITS 1, 5.8S rRNA gene and ITS 2sequences based on distance (Tajima Nei) and Neighbour joining analysis. Numbers above branches represent bootstrap percentages of 1000 replicates.

Similar to the 18S rRNA gene analysis, variation was also observed between isolates of *B. canis vogeli* with the separation of the isolate I2 from all other isolates. Average identity between isolates of *B. canis vogeli* was 99.3 % (Table 10.7). Intra-subspecies variation was also found to exist between *B. canis rossi* isolates (99.8 % identity). No variation was found between isolates of *B. canis canis* (H1 and F1) or *B. gibsoni* (A5 and NC1). Percentage similarity between each of the *B. canis* subspecies ranged from 55.2 – 82.6 % and between species, ranged from 27.2 – 51.4 % (Table 10.8).

	Bcr	**Bcp**	**Bcv**	**Bcc**	***B. gibsoni***	***B. caballi***
Bcr	99.8					
Bcp	81.4	n/a				
Bcv	57.5	59.6	99.3			
Bcc	55.2	56.6	82.6	0.00		
B. gibsoni	27.3	34.6	34.0	31.1	0.00	
B. caballi	27.2	29.8	28.4	20.0	51.4	n/a

Table 10.8

Percentage similarity based on Kimura 2-parameter distance using the ITS 1, 5.8S rRNA and ITS 2 (744bp) (Bcr – *B. canis rossi*, Bcp – *B. canis presentii*, Bcv – *B. canis vogeli*, Bcc – *B. canis canis*)

10.4.4 Amplification, sequencing and phylogenetic analysis of the HSP 70 gene

A partial region of the HSP 70 gene was amplified for the large piroplasm isolates A2, M2, B3 (*B. canis vogeli*, Figure 10.7), SA1, SA4 (*B. canis rossi*) and H2 (*B. canis canis*) and the small piroplasm isolates A5 and NC1 (*B. gibsoni*).

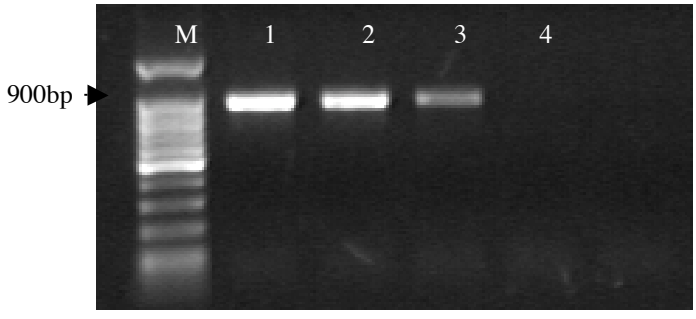


Figure 10.7

Amplification of a partial region (886 bp) of the HSP 70 gene (M – molecular marker, 1 – A2, 2 – M2, 3 – B3, 4 – negative control)

Variation was observed between each of the isolates of *B. canis vogeli* (A2, M2 and B3) with a total of eight variable nucleotide positions found across a 656 bp region of the HSP 70 gene (Figure 10.8).

```
M2              CATGGACAAGTCCACCGGAAAGTCCCAGCAGGTCACCATCACCAACGACAAGGGTCGTCT 420
B3              CATGGACAAGTCCACCGGAAAGTCCCAGCAGGTCACCATCACCAACGACAAGGGTCGTCT 420
A2              CATGGACAAGTCCACCGGAAAGTCCCAGCAGGTCACCATCACCAACGACAAGGGTCGTCT 420
                ************************************************************

M2              CAGCACTGCTGACATTGAGCGTATGGTTGCCGAGGCCGAGAAGTTCAAGGAGGAGGACGA 480
B3              CAGCACTGCTGACATTGAGCGTATGGTTGCCGAGGCCGAGAAGTTCAAGGAGGAGGACGA 480
A2              CAGCACTGCTGACATTGAGCGTATGGTTGCCGAGGCCGAGAAGTTCAAGGAGGAGGACGA 480
                ************************************************************

M2              GACCAGGCGCCAGTGCGTCGAGGCCAAGCACCAACTCGAGAACTACTGCTACAGCATGAA 540
B3              GACCAGGCGCCAGTGCGTCGAGGCCAAGCACCAACTCGAGAACTACTGCTACAGCATGAA 540
A2              GACCAGGCGCCAGTGCGTCGAGGCCAAGCACCAACTCGAGAACTACTGCTACAGCATGAA 540
                ************************************************************

M2              GTCCACCCTGGGCGAAGAGAAGGTCTAAAGAGAAGCTTGACGCTT-CTGAGGTCAGCCAG 599
B3              GTCCACCCTGGGCGAAGAGAAGGTC-AAAGAGAAGCTTGACGCTT-CTGAGGTCAGCCAG 598
A2              GTC-ACCCTGGGCGAAGAGAAGGTC-AAAGAGAAGCTTGTCGCTTTCTGATGTCAGCCAG 598
                ***.********************.*************.*****.****.*********

M2              GCTATGACTGTGATTGAGGACGCCATC-AAGTGGCTCGAGACCAACCAAA-CCGCCACC 656
B3              GCTATGACTGTGATTGAGGACGCCATC-AAGTGGCTCGAGACCAACCAAAACCGCCACC 656
A2              GCTATGACTGTGATTGAGGACGCCATCTAAGTGGCTCGAGACTAACCAAA-CCGCCACC 656
                ***************************.**************.*******.********
```

Figure 10.8

Clustalw alignment of a partial region of the HSP 70 gene of isolates M2, B3 and A2 (variable nucleotide sites are shaded)

Sequencing of isolates A5 and NC1 revealed the presence of mixed DNA template and accurate sequence information could not be obtained for phylogenetic analysis. Sequences obtained for each of the *B. canis* subspecies, along with Genbank sequences for *B. gibsoni, B. bovis* and *T. annulata* were used to conduct a phylogenetic analysis based on 499 base pairs of the HSP 70 gene (Figure 10.9). All three *B. canis* subspecies formed a clade distinct from *B. gibsoni* and *B. bovis*, with strong statistical support (74 % bootstrap support). *Babesia canis rossi* was shown to be the most ancestral subspecies, while *B. canis canis* and *B. canis vogeli* exhibited a closer evolutionary relationship.

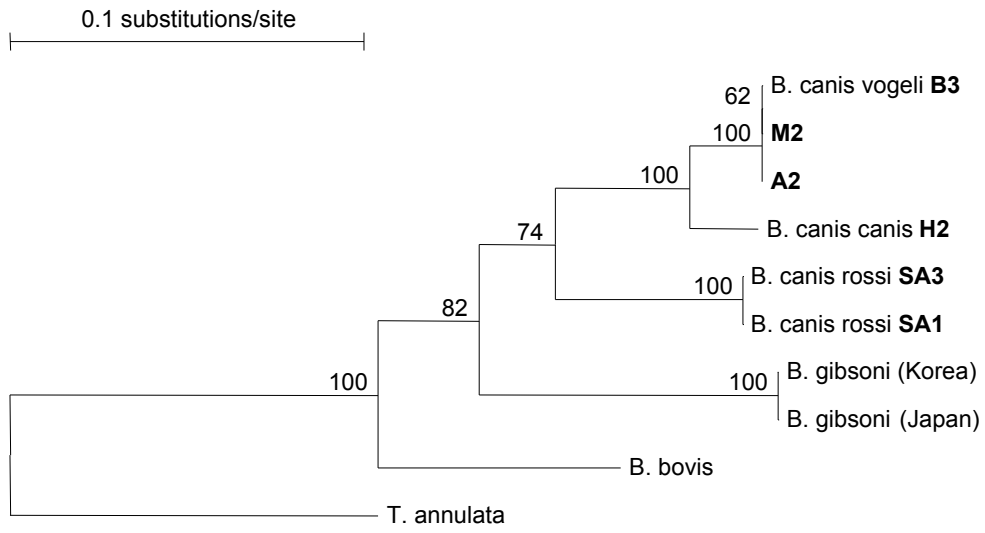


Figure 10.9

Phylogenetic tree constructed using partial HSP 70 gene sequences based on distance (Tajima Nei) and Neighbour joining analysis. Numbers above branches represent bootstrap % of 1000 replicates.

The level of genetic similarity between each of the *B. canis* subspecies was also determined and compared to the level observed between other established piroplasm species (Table 10.9). A similar level of sequence homology was found to exist between *B. canis rossi* and *B. canis canis* and *B. canis vogeli* (89.8 and 89 % respectively) as between *B. canis rossi* and *B. gibsoni* (87 %). Less comparable was the very high sequence homology witnessed between *B. canis canis* and *B. canis vogeli*.

	Bcr	**Bcc**	**Bcv**	***B. gibsoni***	***T. annulata***
Bcr	0.00				
Bcc	89.8	n/a			
Bcv	89.0	96.1	0.00		
B. gibsoni	87.0	83.0	83.5	0.00	
T. annulata	66.6	69.2	70.3	71.2	n/a

Table 10.9

Percentage similarity based on Kimura 2-parameter distance using the HSP 70 gene (499 bp) (Bcr – *B. canis rossi*, Bcv – *B. canis vogeli*, Bcc – *B. canis canis*)

10.5 Discussion

The results of this chapter describe the most comprehensive investigation into genetic variation among the canine piroplasm species to date. This has given greater insight into the distribution of the selected species and reveals the level of intra-species variation using conserved and variable gene loci between isolates from a wide range of geographical locations worldwide. This is the first study to reports the sequencing of the HSP 70 gene for *B. canis vogeli, B. canis canis canis* and *B. canis rossi,* and the ITS 1, 5.8S rRNA gene and ITS 2 for *B. gibsoni*. The following sections will discuss the molecular characterisation of and the levels of genetic variation among the species *B. canis* and *B. gibsoni* and will address the issues of molecular taxonomy and the species concept. Re-classification of current taxonomic anomalies within these species will also be discussed.

It is important to note that due to strict quarantine regulations in Australia, FTA cards were used for the transport, storage and DNA amplification of samples from multiple countries (evaluation of FTA is described in Chapter five). This method however, became a significant limitation during the course of this study. The amplification of target DNA from FTA samples proved problematic due to the preferential amplification of host DNA and contamination with fungal growth due to the high humidity and the difficulty in drying the blood samples obtained from tropical countries such as Malaysia and the Philippines. Designing more specific primers for amplifying target genes of the canine piroplasm species should be considered, including using nested-PCR, to increase the likelihood of amplifying the low DNA template levels of FTA-based samples.

It should also be noted that the use of partial gene sequences was a consequence of problematic amplification and the availability of only partial sequence information on the GenBank database. While levels of genetic variation on the basis of partial sequences may be informative, this may not be an accurate representation of the entire gene and as such, use of complete gene sequences would have greatly reinforced the phylogenetic and

phylogeographical data obtained in this chapter. The following hypotheses discussed with regard to genetic characterisation and phylogeography of the selected canine piroplasm species should be considered as preliminary studies and need to be followed up with comprehensive studies using complete gene sequences of multiple loci before definitive theories can be postulated.

10.5.1 Genetic characterisation and phylogeography of the Babesia canis subspecies

i) *Babesia canis vogeli*

Molecular characterisation of multiple isolates of *B. canis* from different geographic locations within Australia further supports that *B. canis vogeli* is the predominant large canine piroplasm species in Australia (Jefferies *et al.*, 2003) and is likely to be a reflection of the cosmopolitan distribution of the tick vector *R. sanguineus.* The absence of the tick vectors responsible for the transmission of other subspecies in Australia is likely to prevent establishment, however stringent tick control measures should be maintained to prevent exotic canine tick species entering Australia.

Through the use of molecular characterisation, *B. canis vogeli* has also been confirmed to be present within many regions of the world for the first time, suggesting that this subspecies is likely to be the most widely distributed of all the *B. canis* subspecies. The genetic characterisation of large piroplasm isolates from Malaysia, Thailand and the Philippines has been achieved for the first time, providing a greater insight into the canine *Babesia* in south-east Asia. Research into *B. canis* infections in south-east Asia has been extremely limited (Irwin and Jefferies, 2004). While probable cases of *B. canis* infection have been reported in Malaysia (Rajamanickam *et al.*, 1985) and Thailand (Suksawat *et al.*, 2001b) the subspecies present was not determined. It is now confirmed that *B. canis vogeli* is present within multiple south-east Asian countries and again, is likely to be due to the high endemicity of *R. sanguineus* throughout the region.

Before the commencement of this research, limited study had been conducted on canine babesiosis in South America. O'Dwyer *et al.* (2001) reported coinfections of a large intraerythrocytic piroplasm presumed to be *Babesia canis* and *Hepatozoon canis* in Brazil. A more recent study investigated clinical cases of canine babesiosis in Belo Horizonte, Minas Gerais, Brazil, suggesting a high infection rate of this disease in dogs surveyed (Bastos *et al.*, 2004). *Babesia canis* has also been reported in maned wolves (*Chrysocyon brachyurus*) at the Sorocaba Zoo, Brazil (Nunes, 1989). While canine *Babesia* spp. have been previously documented to exist in South America, this study, along with Passos *et al.* (2005) describe the molecular characterisation of these piroplasms for the first time. All isolates were determined to be *B. canis vogeli,* a subspecies now confirmed to be present in both Brazil and Uruguay. *Rhipicephalus sanguineus*, the principal vector of *B. canis vogeli,* has been reported to exist in both Brazil and Uruguay. This tick was originally introduced from the Afrotropical region to Brazil, where it has been reported to be an increasingly widespread pest in urban environments (Evans *et al.*, 2000; Szabo *et al.*, 2001). Likewise, *R. sanguineus* has been reported in Uruguay (Rodriguez and Lazaro, 1954; Venzal *et al.*, 2003). This tick species has also been found in Mexico (Cruz-Vazquez and Garcia-Vazquez, 1999), Panama (Miller *et al.*, 2001), Venezuela (Unver *et al.*, 2001) and Argentina (Guglielmone *et al.*, 1991; Ruiz *et al.*, 2003), which suggests that *B. canis vogeli* may also be widespread throughout Central and South America.

The existence of *B. canis* has previously been reported within Israel (Baneth *et al.*, 1998) and sequencing of a 270 bp region of the 18S rRNA gene suggested that *B. canis vogeli* is the subspecies present (Baneth *et al.*, 2004). In the present study, sequencing a larger region of the 18S rRNA gene from isolates from four separate geographic regions has confirmed that *B. canis vogeli* is indeed the subspecies present within Israel. Isolates from Israel showed greatest homology to an isolate from Egypt that is considered the type specimen for *B. canis vogeli* (Passos *et al.*, 2005). A possibly geographic correlation may exist between isolates from Africa and the Middle East. *Babesia canis vogeli* has also been reported in South

Africa (Matjila *et al*., 2004) and Sudan (Oyamada *et al*., 2005), however the small size of the partial sequences amplified prevented its inclusion in the analysis performed in this chapter.

As a result of this study, it should also be recognized that three distinct genotypes of the subspecies *B. canis vogeli* occur on the basis of the 18S rRNA gene and is a possible reflection of their different geographical origins. The most common genotype described was in isolates from Australasia and Brazil and was shown to be ancestral to other isolates. A second from dogs in Egypt and Israel and a third genotype was found to be present in Europe, USA and Uruguay. Previous studies have recognized the existence of low levels of genetic variation between isolates of the *B. canis* subspecies (Zahler *et al.,* 1998; Caccio *et al*., 2002; Passos *et al*., 2005) however these studies did not correlate this variation to possible phylogeographical relationships between isolates. Also, the possibility of differences in biology or pathogenicity between each of these genotypes requires further study.

It is postulated that *B. canis vogeli* may have originated from Asia and may be correlated to the geographical origins of the domestic dog. A recent study suggested that the dog originated from eastern Asia (Savolainen *et al*, 2002). The similarity in genotype of *B. canis vogeli* isolates from Asia and Australia may relate to early dog movement, notably dingoes (*Canis familiaris dingo*) from Asia to Australia. *Babesia canis* has also been reported in dingoes within Australia (Callow, 1984; Jefferies, 2001) and molecular characterisation is necessary in order to determine the genotype present within these wild canines. The reason that isolates from Brazil are genetically distinct from Uruguay and similar to isolates from the Australasian region remains unknown. Further study investigating *B. canis vogeli* isolates from additional geographical locations, in addition to co-evolutionary relationships with dogs and tick vectors is necessary before definitive conclusions on the phylogeography of this subspecies can be determined.

It is interesting to hypothesise that the clustering of the isolate from Uruguay with sequences from Spain and France may be a reflection of the European colonization of this country. It is conceivable that immigrants from Spain or Portugal may have brought with them the first *Babesia canis vogeli* infected dogs or ticks to Uruguay. A similar situation may have also occurred in the USA. Unfortunately, isolates from Uruguay and Spain could not be amplified on the basis of the ITS 1, 5.8S rRNA gene to confirm the existence of distinct genotypes.

ii) *Babesia canis canis*

Distinct genotypes were also observed between different isolates of *B. canis canis*, supporting the genetic variation observed in previous studies (Caccio *et al.*, 2002; Duh *et al.*, 2004). In contrast to *B. canis vogeli*, these genetic differences did not reflect the geographical origin of the isolate. A possible explanation for the mixture of genotypes throughout Europe is a reflection of recent increases in dog movement throughout countries of the European Union, with the introduction of travel schemes such as PETS (Pet Travel Scheme) (Shaw *et al.*, 2001b). The existence of allopatric (non-overlapping) populations may have originally enabled distinct genotypes to develop, however subsequent movement of dogs between various countries could have lead to a mixture of genotypes existing in the same geographic location. Only small partial regions of the 18S rRNA gene could be used to investigate isolate heterogeneity and further research using the complete or near complete 18S rRNA gene, in addition to other gene loci is necessary to confirm the existence of genotypes within *B. canis canis* and what significance this variation may have on phylogeography and biological differences.

iii) *Babesia canis rossi*

This study has revealed very limited genetic variation witnessed among different isolates of *B. canis rossi*. Until recently, *B. canis rossi* was believed only to be present within South Africa, however with its discovery in Sudan (Oyamada *et al.*, 2005), this subspecies may be more widely distributed throughout Africa. Further investigation into the distribution of this

subspecies is necessary to determine how widespread *B. canis rossi* is in the African continent and whether it exists in other countries outside Africa. Recently described in Australia was a case of a dog being imported from South Africa via Hong Kong that was found to be infected with *B. canis rossi* (Ainslie Brown, AQIS, pers. com) suggesting that sporadic infections with this subspecies may occur outside of Africa and should be considered when diagnosing canine piroplasmosis worldwide.

10.5.2 Genetic characterisation and phylogeography of Babesia gibsoni

This study has further characterised isolates of *B. gibsoni* from Australia. While, isolates of this species in Australia have previously been characterised on the basis of the 18S rRNA (Muhlnickel *et al*., 2002; Jefferies *et al*., 2003), only small partial regions of the gene were sequenced, limiting accurate comparisons to other isolates. This study, in contrast, has sequenced isolates from two separate states, including an isolate from New South Wales for the first time, on the basis of a larger partial region of the 18S rRNA gene. In addition, the complete ITS 1, 5.8S rRNA gene and ITS 2 sequences were determined for an isolate from Victoria. This is the first reported sequencing of these combined loci for *B. gibsoni*.

Also described for the first time, is the presence of *B. gibsoni* in Singapore, Taiwan and Hong Kong, on the basis of the molecular detection and characterisation of the 18S rRNA gene, in addition to further confirming the genotype of this species in Malaysia and Sri Lanka. (Zahler *et al*., 2000c). Unfortunately DNA of small piroplasm isolates from Brazil (B6-9) could not be amplified and while the existence of small piroplasms in Brazil should now be recognized, the species and/or genotype remains unknown and is currently the subject of further investigation.

Using the 18S rRNA gene, two main genotypes of *B. gibsoni* were found to exist, with the notable phylogenetic separation of the *B. gibsoni* isolate from Spain from all other isolates. The distinction of *B. gibsoni* from Spain from other isolates was first reported by Criado-

Fornelio *et al*. (2003d), who suggested that this species may have originated from Asia and developed into two sister lineages. *Babesia gibsoni* has also been reported to exist in Egypt, Nigeria and Mali (Yamane *et al*., 1993), however no isolates from Africa have ever been molecularly characterised. It is important to characterise isolates of small canine piroplasms from Africa before a hypothesis on the evolution of *B. gibsoni* can be proposed. It has also been suggested that the genotypes of *B. gibsoni* should not be taxonomically elevated (Criado-Fornelio *et al*., 2003d). Further research into pathogenesis, geographic distribution and tick vectors of each genotype is necessary before the significance of the genetic variation observed is better understood and whether taxonomic revision is appropriate.

The genetic homology that exists on the basis of the 18S rRNA gene between isolates of *B. gibsoni* from Australia and certain regions of the USA and Asia is of particular interest with reference to infections of this species in fighting dog breeds (refer to Chapter seven). Identical sequences of the ITS 1, 5.8S rRNA gene and ITS 2 were also identified for *B. gibsoni* isolates from Australia and the USA. As is presumed with all the piroplasmid species, the sexual stage of the lifecycle occurs within the tick vector. Sexual reproduction allows for genetic recombination to occur and the production of genetic heterogeneity within a population. If the tick vector was absent during the transmission of *B. gibsoni*, as is suggested to occur in populations of fighting dogs (Macintire *et al*., 2002; Birkenheuer *et al*., 2003b; Matsuu *et al*., 2004a), then it would be expected that a clonal lineage of these protozoa would develop. This indeed may be the case in fighting dog populations from Australia (Chapter seven), certain regions of Japan and the USA. The use of only partial gene sequences for analysis and the conserved nature of the selected loci are perhaps too conserved to draw any definitive conclusions and the sequencing of much less conserved loci is suggested. The absence of genetic variation within *B. gibsoni* isolates from Australia, Japan and certain states of the USA may also simply be a reflection of the recent spread of this parasite to these countries, not allowing sufficient time for any genetic variation to develop.

10.5.3 Molecular taxonomy; defining species level classification

The problematic concept of defining species level categorization has plagued taxonomists throughout history and remains a contentious issue. The traditional concept, that 'species are groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups' (Kunz, 2002), is even more difficult to apply to the protozoa which often have complex life cycles, involving both asexual and sexual reproduction. As the concept of a species is an artificial construct, created as a means of convenient categorization, a universal description should not be applied to all taxa rather individual definitions of a species should be devised for separate groups of organisms. General criteria for describing a new species should however be conformed to and follow the guidelines established by the International Code of Zoological Nomenclature (ICZN) (Ride *et al.*, 1999). Criteria for describing a new species have been postulated for other apicomplexans such as *Cryptosporidium*, including parasite morphology, host specificity and genetic characterisation (Xiao *et al.*, 2004)

Uilenberg *et al.* (2004) argued that current molecular taxonomy using gene sequences, rather than entire genomes, has lead to premature changes in classification systems. They suggested that polyphasic taxonomy, based upon both genotype and phenotype, should be considered before making any new taxonomic changes. Within the piroplasms, overzealous naming of species on the basis of molecular characterisation, such as in the case of the taxonomic description of *T. annae* (Zahler *et al.*, 2000b) has been considered inappropriate (Goethert and Telford, 2003; Reichard *et al.*, 2005). Baneth *et al.* (2004) chose a far more cautious approach when taxonomically describing *B. canis presentii*.

Uilenberg *et al.* (2004) also suggested that molecular-based taxonomy can lead to repeated taxonomic changes and subsequently produce confusion rather than clarification. In contrast to this argument, it should be noted that taxonomic changes were far more confusing during the pre-molecular taxonomy era, as for example, since the first description of *B. canis*, at

least twelve separate species names have been given to this species before the introduction of molecular characterisation (Levine, 1988). Since the molecular characterisation of this species, no taxonomic changes have been made. Such complex pre-molecular taxonomy is common among many piroplasmids. While it is important to consider all available information, both genotypic and phenotypic, before making changes to the taxonomy, overly cautious attitudes by Uilenberg *et al.* (2004) and Goethert and Telford (2003) hinder progressive, yet systematic changes in the taxonomic classification of the piroplams.

This chapter has also shown that the level of genetic variation observed between established *Babesia* species on the basis of the 18S rRNA gene, ITS 1, 5.8S rRNA gene and ITS 2 is similar to that which exists between each of the *B. canis* subspecies. For example, the level of intra-subspecies genetic variation on the basis of the 18S rRNA gene for *B. canis vogeli* and *B. canis canis* is similar to the intra-species variation observed in isolates of *B. gibsoni*. Also for the first time also provides further support with phylogenetic analyses based on a partial region of the HSP 70 gene. These results further support the suggestion that each of the *B. canis* subspecies should be elevated to species level classification (Uilenberg *et al.*, 1989; Zahler *et al.*, 1998; Carret *et al.*, 1999). Schnittger *et al.* (2003) proposed that an isolate should be defined as a new species if the genetic identity using the 18S rRNA gene is lower than 99.3 % for *Theileria* and 96.6% for *Babesia* on the basis of sheep and goat piroplasms, a feature also observed between each of the *B. canis* subspecies. Other studies have delineated piroplasm species level classification using the internal transcribed spaces (ITS 1 and 2) and the intervening 5.8S rRNA gene (Zahler *et al.*, 1998; Holman *et al.*, 2003).

Phylogenetic analysis in the present study also revealed that two unnamed *Babesia* species, one identified from Red-cheeked sousliks (*Citella erythrogenys*) in China (Zamoto *et al.*, 2004) and the second from Bandicoot rats (*Bandicota indica*) in Thailand (Dantrakool *et al.*, 2004) are closely related to the *B. canis* subspecies. *Babesia kiwiensis* also shows a phylogenetic affiliation to the *B. canis* group. Further study needs to be carried out on the

phylogenetic relationships between each of these species on the basis of multiple gene loci. Previous studies into the phylogenetic relationships of the *B. canis* subspecies have found that each of the subspecies cluster together in a monophyletic clade, separate to all other *Babesia* spp. (Carret e*t al.*, 1999; Criado-Fornelio *et al.*, 2003b; Baneth *et al.*, 2004; Caccio *et al.*, 2002) and may have contributed to each of the subspecies not being recognised as different species. It therefore becomes important to be able to define species level classification among the piroplasms. Species level classification on the sole basis of a certain level of genetic variation may be misleading as some separate species may possess identical genetic sequences in some genomic regions and not in others (Xiao *et al.*, 2004). No stipulations are given by the ICZN for the description of new species on the basis of molecular characterisation (Ride *et al.*, 1999).

Significant differences between *B. canis canis*, *B. canis vogeli*, *B.canis rossi* and *B. canis presentii* on the basis of one or more of, pathogenesis, vector specificity and genetic variation suggests that subspecies level classification for *B. canis* is inappropriate and it may be deemed necessary to elevate each to assume species level status. Thus taxonomic classification can be based on both molecular and biological characteristics.

10.5.4 Proposed re-classification of the B. canis subspecies, including the re-description of B. canis (Piana and Galli-Valeria, 1895), B. rossi (Wenyon, 1926) and B. vogeli (Reichenow, 1937)

Babesia canis was first described by Piana and Galli-Valeria (1895) and subsequently all large piroplasms (3 – 5 □m) found in dogs were classified within this species. It then became evident that differences in vector specificity and cross-immunity existed between different isolates of this species, leading to the description of three *B. canis* subspecies by Uilenberg *et al.* (1989). It is interesting to note that Uilenberg *et al.* (1989) actually suggested that each of the proposed *B. canis* subspecies were likely to be separate species but chose to define them as subspecies on the simple basis of convenience rather than

consistency within taxonomic procedures. Different disease pathologies were also described for each subspecies (Irwin and Hutchinson, 1991; Schetters *et al*., 1997b). *Babesia canis canis*, *B. canis vogeli* and *B. canis rossi* were each then characterised on the basis of the 18S rRNA gene (Carret *et al*., 1999) and the ITS 1, 5.8S rRNA gene and ITS 2 (Zahler *et al*., 1998), confirming the separation of each of these subspecies and allowed for further speculation that a species level of categorization may be more appropriate. Differences between *B. canis canis* and *B. canis rossi* have also been suggested at a genomic level, with respective genome sizes estimated to be 14.5 Mbp and 16 Mbp (Depoix *et al*., 2002) which may further suggest the existence of separate species. Further support for the elevation of each of the *B. canis* subspecies to species level classification has been proved by the genetic characterisation and phylogenetic studies described in this chapter.

The additional subspecies, *B. canis presentii* was later described as a piroplasm of cats (Baneth *et al*., 2004) and was described as having merozoites and trophozoites that were morphologically smaller than *B. canis canis*. Further information regarding host specificity (can this subspecies infect dogs?), pathogenicity and molecular characterisation of multiple genes is required before it can be concluded whether *B. canis presentii* is indeed a separate species or simply a subspecies of *B. canis*. This is combined with current phylogenetic ambiguity of this subspecies when comparing 18S rRNA gene and ITS based analysis, and suggest that no taxonomic changes be made to this subspecies until further study is conducted.

It is proposed that each of the *B. canis* subspecies (excluding *B. canis presentii*) should assume a species level of classification on the basis of the following six criteria, which should be considered when describing any new species of piroplasm:

i) Host

ii) Vector specificity

iii) Morphology

iv) Pathogenesis

v) Genetic characterisation

vi) Geographic distribution

The proposed taxonomic changes are as follows:

- *Babesia canis (Piana and Galli-Valeria, 1895)*

Host/s Dogs, foxes, cats and horses

Vector *Dermacentor reticulatus*

Pathogenicity Moderate disease

Babesia canis presentii (Baneth *et al*., 2004)

Host Cats

Vector unknown

Pathogenicity unknown

- *Babesia vogeli (Reichenow, 1937)*

Host Dogs, possibly other Canidae

Vector *Rhipicephalus sanguineus*

Pathogenicity Mild to moderate disease

- *Babesia rossi ([Nutall, 1910], Wenyon, 1926)*

Host Dogs, Jackals

Vector *Haemaphysalis leachi*

Pathogenicity Highly virulent, moderate to severe haemolytic disease

10.5.5 Conclusions

An attempt has been made to clarify the species concept among the canine and related piroplasms using molecular characterisation. The level of genetic variation distinguishing

species is dependent upon the group of piroplasms investigated and perhaps more specifically the gene loci used for analysis. While the importance of defining genetic variation among species and between species is of great significance, a universal species concept remains elusive. It is therefore imperative that criteria for describing new piroplasm species be established to allow for less confusion when describing new isolates and it is suggested that the host, vector specificity, morphology, pathogenesis, genetic characterisation and geographic distribution are considered as potential criteria. This chapter has also revealed that a number of genotypes are likely to exist within each canine piroplasm species, however the taxonomic or pathological significance of these genotypes is yet to be determined. Further investigation into the phylogeography of the canine piroplasms using less conserved gene loci may allow for a better understanding of the complex epidemiology of these protozoa.

CHAPTER ELEVEN

Phylogenetic and taxonomic status of the order Piroplasmida: Defining family level classification

11.1 Introduction

Traditional schemes of taxonomic classifications have, in the past, concentrated on phenotypic features such as life cycle and morphological characteristics as well as host-parasite relationships, however the classification of many taxa is currently under review with the introduction of genetic sequencing and phylogenetic-based analysis. This is a common feature of the classification of most protozoa belonging to the phylum Apicomplexa (Cavalier-Smith, 1993; Escante and Ayala, 1995; Bernhard *et al*., 2001; Tenter *et al*., 2002; Xiao *et al*., 2004), including the order Piroplasmida (Allsopp *et al*., 1994; Reichard *et al*., 2005).

DNA sequencing of target genes has become a highly effective means of characterising established species within the order Piroplasmida and has also given rise to the discovery of multiple new species and genotypes (Zahler *et al.,* 2000b; Dantrakool *et al*., 2004). While the use of molecular technology has allowed for increased diagnostic accuracy, it has also led to some confusion over the taxonomic position of many species of piroplasm. For example, certain species initially described as belonging to the genus *Babesia* have later been found to be more genetically similar to *Theileria* species, suggesting that the taxonomy may need to be clarified. It is now reported that both the *Theileria* and *Babesia* are paraphyletic taxa (Allsopp *et al*., 1994; Zahler *et al*., 2000b; Reichard *et al*., 2005).

There is also current debate over the phylogeny and taxonomy of the *B. microti* group of piroplasms, described as the Archeopiroplasmids (Criado-Fornelio *et al.*, 2003b) which include the species, *T. annae, B. felis* and *B. leo.* While it has been suggested that this group of piroplasms is ancestral to both the genera *Babesia* and *Theileria*, their taxonomic position has not been determined with absolute certainty. Also suffering taxonomic ambiguity are the Western USA *Babesia* spp. described by Kjemtrup *et al.* (2000b), later referred to as the Prototheilerids (Criado-Fornelio *et al.*, 2003b). It is not clear whether both the Archeopiroplasmid and Prototheilerid groups should be allocated to a separate genus and also possibly to a new family group. Re-defining family level classification within the Order Piroplasmida is therefore overdue and needs to be determined to limit current taxonomic confusion.

One of the problematic features of current phylogeny and taxonomy on the basis of gene sequences is the predominant use of the 18S rRNA gene. While the 18S rRNA gene is commonly used as a 'molecular clock' for determining rates of evolution among various organisms due to its highly conserved nature, analysis using a single gene locus is simply a reflection of evolution of that gene. Phylogeny based on other gene loci, such as the less conserved ITS regions and the intervening 5.8S rRNA gene may offer greater insight into the evolutionary relationships of the piroplasmids. Another group of genes that have phylogenetic potential are the HSP 90 and HSP 70 genes that encode for the heat shock proteins, synthesized as a response to an elevation in temperature in all organisms ranging from archaebacteria to plants and animals (Lindquist and Craig, 1988; Hendrick *et al.*, 1995). The highly conserved nature of these genes, therefore make them a desirable candidate for determining phylogenetic relationships between selected taxa. Multiple species of piroplasm have been characterised on the basis of the HSP 70 gene (Daubenberger *et al.*, 1997; Ruef *et al.*, 2000; Yamasaki *et al.*, 2002), however few studies have used this gene to infer evolutionary relationships among the Piroplasmida.

Phylogenetic analysis of combined gene loci has been suggested as a more accurate method for determining the evolution of a species, rather than just of a single gene, and has been used for a variety of organisms (Devulder *et al*., 2005; Hypsa *et al*., 2005). Multi-locus analyses can also accurately reflect the evolution of entire genomes (Zeigler, 2003).

By incorporating new gene sequences of *B. canis* and *B. gibsoni* derived earlier in this study (refer to Chapter ten), the phylogenetic relationships and taxonomy of the Piroplasmida will be investigated using multiple, including combined, gene loci analysis.

11.2 Aims

i. To determine the phylogenetic relationships among members of the Piroplasmida on the basis of the 18S rRNA gene, ITS 1, 5.8S rRNA gene and HSP 70 gene.
ii. To investigate the phylogenetic relationships of the Piroplasmida using a combined gene loci approach.
iii. To investigate the phylogenetic relationships between the Piroplasmida, Haemosporida and other Apicomplexan taxa on the basis of the 18S rRNA gene, ITS 1, 5. 8S rRNA gene and HSP 70 gene.
iv. To re-evaluate the taxonomic status of members of the Piroplasmida, including redefining family level classification.

11.3 Material and Methods

11.3.1 DNA sequences

All available piroplasm sequences for the 18S rRNA gene (Table 11.1) ITS 1, 5.8S rRNA gene, ITS 2 (Table 11.2), and HSP 70 gene (Table 11.3) were accessed from the GenBank database (accessed 02/2005). Additional sequences were included for analysis from this study (refer to Chapter ten).

Piroplasmida sp.	**Host**	**Location**	**Accession No**
Babesia sp. (Bandicoot rat)	Bandicoot rat	Thailand	AB053216
Babesia bicornis	Black Rhinoceros	Tanzania	AF419313
Babesia bigemina	Cow	Mexico	X59607
Babesia bovis	Cow	Portugal	AY150059
Babesia caballi	Horse	Spain	AY309955
Babesia canis canis	Dog	Croatia	AY072926
Babesia canis presentii	Cat	Israel	AY272047
Babesia canis vogeli	Dog	Okinawa, Japan	AB083374
Babesia canis rossi	Dog	South Africa	L19079
Babesia divergens	Reindeer	USA	AY098643
Babesia divergens	Rabbit	Massachusetts, USA	AY144688
Babesia(Theileria) equi (dog)	Dog	Spain	AY150064
Babesia(Theileria) equi	Horse		Z15105
Babesia felis	Cat	South Africa	AF244912
Babesia gibsoni	Dog	Spain	AY278443
Babesia leo	Lion	South Africa	AF244911
Babesia microti	*Ixodes ovatus*	Hyogo, Japan	AB070506
Babesia odocoilei	Reindeer	Wisconsin, USA	AY237638
Babesia ovata	Cow	Korea	AY081192
Babesia ovis	Goat	Spain	AY150058
Babesia rodhaini	Mouse		AB049999
Babesia sp. Akita		Japan	AY190123
Babesia sp. Coco	Dog	North Carolina, USA	AY618928
Babesia sp. EU (*Babesia venatorum*)	Human	Europe	AY046575
Babesia sp. Fukui		Japan	AY190124
Babesia sp. GA	Dog	Georgia, USA	AF396748
Babesia sp. MO1	Human	Missouri, USA	AY048113
Babesia sp. RD1	Reindeer	USA	AF158711
Babesia sp. IoRK/HM101		Japan	AB070506
Babesia sp (Spanish dog)	Dog	Spain	AF188001
Babesia sp. WA1 (*Babesia duncani*)	Human	California, USA	AY027816
Babesia sp. Xinji	Red-cheeked souslik	Xinjiang, China	AB083376

Theileria annulata			M64243
Theileria bicornis	Black Rhinoceros	South Africa	AF499604
Theileria buffeli	Cow	Australia	AF236094
Theileria cervi	White-tailed deer	Oklahoma, USA	AF086804
Theileria lestoquardi	Sheep	unknown	AF081135
Theileria mutans	Cow	Kenya	AF078815
Theileria parva	Buffalo	Kenya	AF013418
Theileria sergenti			AB016074
Theileria sp	Sika deer	unknown	AB012199
Theileria taurotragi	Cow		L19082
Theileria velifera	Cow	Tanzania	AF097993
Theileria youngi	Dusky-footed woodrat	California, USA	AF245279
Cytauxzoon felis	Cat	Oklahoma, USA	AF399930
Cytauxzoon manul	Pallas cat	Mongolia	AY485691
Cytauxzoon sp. Spain	Cat	Spain	AY309956
Cytauxzoon sp. Iberian Lynx	Iberian Lynx	Spain	AY496273
Babesia conradae (CA dog)	Dog	California, USA	AF158702
Piroplasmida gen sp. BH1	Bighorn sheep	California, USA	AF158708
Piroplasmida gen sp. FD1	Fallow deer	USA	AF158707
Piroplasmida gen sp. CA1		California, USA	AF158703
Plasmodium berghei			M14599
Plasmodium cynomolgi		Sri Lanka	L08241
Plasmodium vivax	Human		AY625607
Plasmodium falciparum	Human		M19172
Isospora suis	Pig		U97523
Eimeria maxima	Chicken		U67117
Toxoplasma gondii	Cat		U03070
Cryptosporidium parvum	Pig	China	DQ060424
*Stylonychia lemnae**		China	AJ310496
*Stylonychia pustulata**			X03947

*denotes outgroup species

Table 11.1

Complete 18S rRNA gene sequences of piroplasm species from the GenBank database.

Piroplasmida sp.	Host	Origin	Accession No
Babesia caballi	Horse	Namibia	AF394536
Babesia canis rossi	Dog	South Africa	AF394535
Babesia canis canis	Dog	France	This study (Chapter ten)
Babesia canis vogeli	Dog	Australia	This study (Chapter ten)
Babesia canis presentii	Cat	Israel	AY272048
Babesia conradae	Dog	California, USA	AY965739
Babesia duncani	*Human*	Washinton, USA	AY965741
Babesia felis	*Cat*	South Africa	AY965742
Babesia microti	Syrian hamster	Japan	AB112337
Babesia muratovi	Mouse	Tajikistan	AF510202
Babesia odocoilei	Reindeer	USA	AY345122
Babesia sp. BH1	Bighorn Sheep	USA	AY965735
Babesia sp. (California RD61)	Reindeer	USA	AY339746
Babesia sp. FD1	Fallow deer	USA	AY965737
Babesia sp. MD1	Mule deer	USA	AY965736
Cytauxzoon felis	Cat	Texas, USA	AY531524
Theileria parva	Cow	South Africa	AF086733
Babesia gibsoni	Dog	Australia	This study (Chapter ten)
*Plasmodium vivax**	Human		AF316893
*Plasmodium falciparum**	Human		U48228
*Toxoplasma gondii**	Cat		L49390
*Cryptosporidium parvum**	Cow		AF040725
*Stylonychia lemnae**			AF508773

*denotes outgroup species

Table 11.2

ITS2 - 5.8S sequences for various piroplasm species from the GenBank database.

Piroplasmida sp.	Host	Location	GenBank No
Babesia bovis	Cow		AF107118
Babesia gibsoni	Dog	Korea	AB083513
Babesia microti	Human		U53448
Babesia rodhaini			AB103587
Theileria annulata			J04653
Theileria sergenti	Cow		D12692
Theileria parva	Cow		U40190
Babesia canis vogeli	Dog	Australia	This study (Chapter ten)
Plasmodium falciparum	Human		AB050740
Plasmodium cynomolgi			M90978
Plasmodium berghei			L40815
Toxoplasma gondii	Cat		AF045559
Eimeria maxima			Z46964
Cryptosporidium parvum	Cow	Hungary	AJ310881
Cryptosporidium baileyi		Hungary	AJ310880
*Stylonychia lemnae**			AF227962

*denotes outgroup species

Figure 11.3

HSP 70 sequences for various piroplasm species from the GenBank database.

10.3.2 Sequence alignment and phylogenetic analysis

Each group of sequences was aligned using Clustal W (Thompson *et al*., 1994) and further edited manually using McClade v. 3 (Maddison and Maddison, 1992). Phylogenetic analysis was conducted using TREECON version 1.3b (Van de Peer and De Wachter, 1993) (distance-neighbour joining) and MEGA v. 3 (Maximum parsimony) (Kumar *et al*., 2004). Distance analysis was estimated on the basis Kimura (1980), Tajima and Nei (1984) and Galtier and Gouy (1995) algorithims and tree topologies were inferred using Neighbour-joining (Saitou and Nei, 1987). Statistical support for each tree was determined by using at least 1000 bootstrap replicates. *Stylonychia lemnae*, a free-living ciliate (Oxytrichidae) was used as an outgroup species. A combined gene loci analysis was also conducted using both partial HSP 70 and 18S rRNA gene sequences.

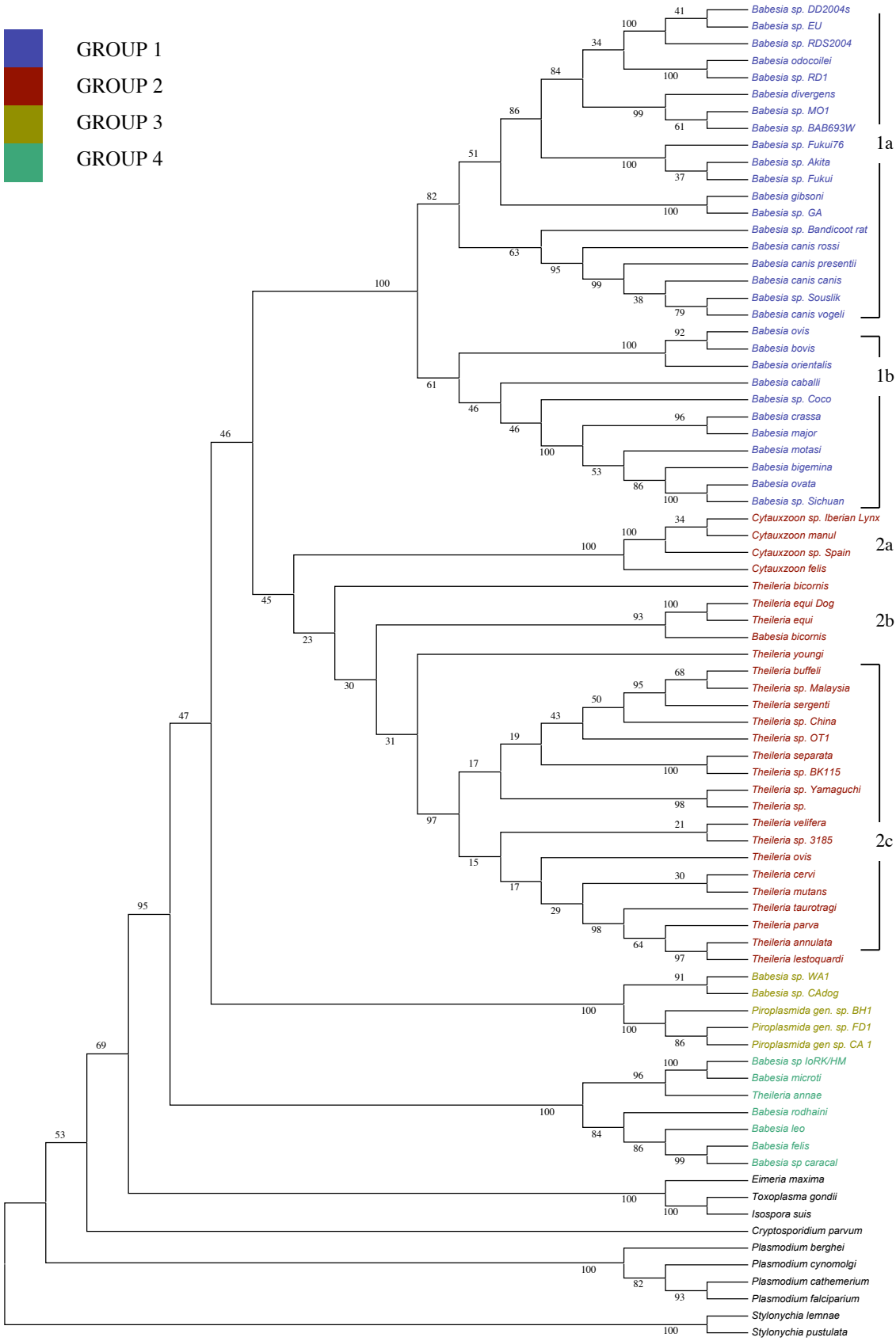
11.4 Results

11.4.1 Phylogeny of the Piroplasmida – 18S rRNA gene analysis

The separation of the Piroplasmida species into four distinct clades (Groups 1-4[6]) on the basis of the 18S rRNA gene was produced for maximum parsimony analyses, with strong bootstrap support for Groups 1, 3 and 4 (Figure 11.1). Group 4 was most distantly related to all other piroplasmid groups and included the *B. microti* related spp. Group 3, containing the *Babesia* sp. WA1 related spp., was ancestral to both Groups 1 (*B. bovis* and *B. canis* related spp.) and 2 (*T. parva* and *Cytauxzoon* related spp.). Distance based analysis also produced four distinct piroplasmid clades with strong bootstrap support (Figures 11.2, 11.3). The position of Groups 3 and 4 was shown to be influenced by the outgroup spp. used. When all outgroup spp. were included in the analysis (Figure 11.2), both Groups 3 and 4 clustered with Group 2 to form a separate clade to Group 1. A second analysis (Figure 11.3), using only *Eimeria, Isospora* and *Toxoplasma* as outgroup spp. gave a tree structure more similar to the maximum parasimony tree, with both Groups 3 and 4 placed ancestral to Groups 1 and 2.

The existence of additional clades within Groups 1 and 2 allowed for further sub-categorization for both maximum parasimony (Figure 11.1) and distance (Figures 11.2, 11.3) analysis. Group 1 was divided into two subgroups, 1a that includes *B. canis* and *B. odocoilei* and 1b that includes *B. bovis* and *B. bigemina*. Group 2 was divided into 2a (the *Cyauxzoon* spp. and *B. bicornis*) and 2b (the *T. equi* related spp.) both of which were ancestral to 2c (the *T. parva* related spp.) *Theileria youngi* did not cluster with any of these subgroups but was ancestral to group 2c. Limited bootstrap support was produced for the phylogenetic position of 2a, 2b and *T. youngi* using maximum parsimony.

[6] Group notation is consistently used for the same groups of species for all analyses throughout section 11.4.


GROUP 1
GROUP 2
GROUP 3
GROUP 4
Babesia sp. DD2004s
Babesia sp. EU
Babesia sp. RDS2004
Babesia odocoilei
Babesia sp. RD1
Babesia divergens
Babesia sp. MO1
Babesia sp. BAB693W
Babesia sp. Fukui76
Babesia sp. Akita
Babesia sp. Fukui
Babesia gibsoni
Babesia sp. GA
Babesia sp. Bandicoot rat
Babesia canis rossi
Babesia canis presentii
Babesia canis canis
Babesia sp. Souslik
Babesia canis vogeli
Babesia ovis
Babesia bovis
Babesia orientalis
Babesia caballi
Babesia sp. Coco
Babesia crassa
Babesia major
Babesia motasi
Babesia bigemina
Babesia ovata
Babesia sp. Sichuan
Cytauxzoon sp. Iberian Lynx
Cytauxzoon manul
Cytauxzoon sp. Spain
Cytauxzoon felis
Theileria bicornis
Theileria equi Dog
Theileria equi
Babesia bicornis
Theileria youngi
Theileria buffeli
Theileria sp. Malaysia
Theileria sergenti
Theileria sp. China
Theileria sp. OT1
Theileria separata
Theileria sp. BK115
Theileria sp. Yamaguchi
Theileria sp.
Theileria velifera
Theileria sp. 3185
Theileria ovis
Theileria cervi
Theileria mutans
Theileria taurotragi
Theileria parva
Theileria annulata
Theileria lestoquardi
Babesia sp. WA1
Babesia sp. CAdog
Piroplasmida gen. sp. BH1
Piroplasmida gen. sp. FD1
Piroplasmida gen sp. CA 1
Babesia sp IoRK/HM
Babesia microti
Theileria annae
Babesia rodhaini
Babesia leo
Babesia felis
Babesia sp caracal
Eimeria maxima
Toxoplasma gondii
Isospora suis
Cryptosporidium parvum
Plasmodium berghei
Plasmodium cynomolgi
Plasmodium cathemerium
Plasmodium falciparium
Stylonychia lemnae
Stylonychia pustulata
1a
1b
2a
2b
2c

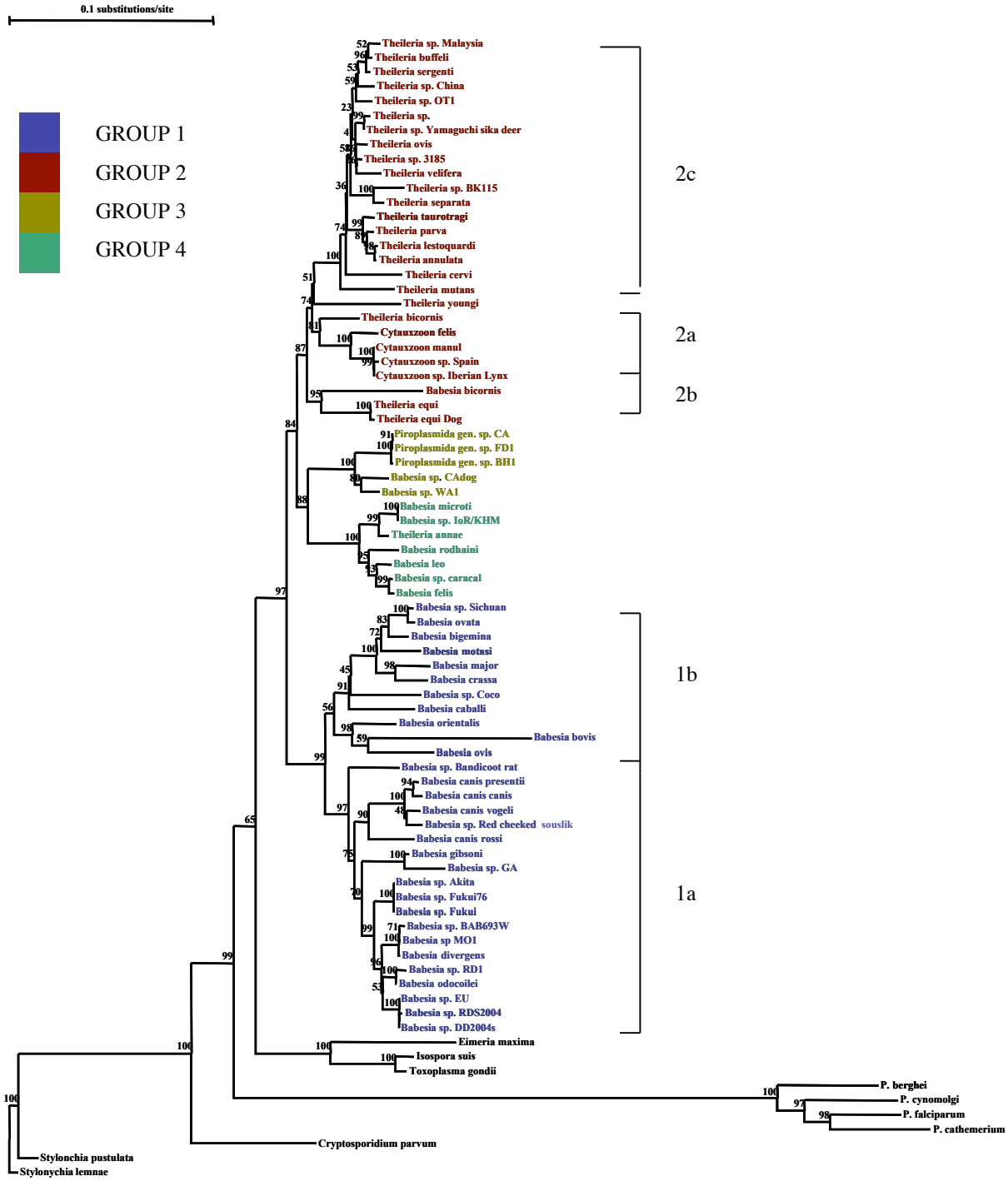


Figure 11.1 (previous page) Phylogenetic tree constructed using a partial 18S rRNA gene sequences based on Maximum Parsimony analysis. Numbers above branches represent bootstrap percentages of 1000 replicates.

Figure 11.2 (this page) Phylogenetic tree constructed using a partial 18S rRNA gene sequences based on distance (Tajima Nei) and Neighbour joining analysis. Numbers above branches represent bootstrap percentages of 1000 replicates.

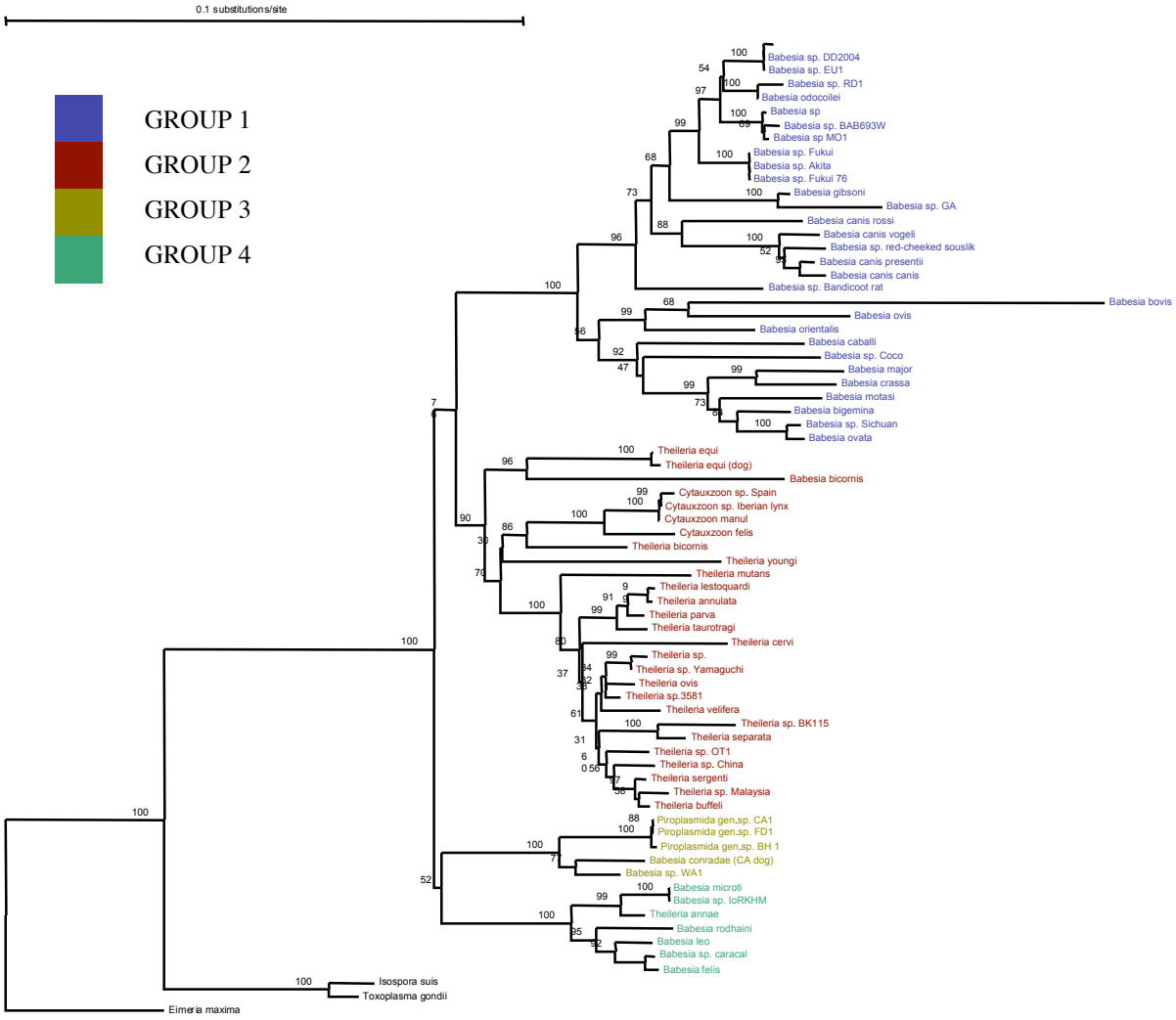


Figure 11.3

Phylogenetic tree constructed using a partial 18S rRNA gene sequences (excluding *Plasmodium* spp) based on distance (Tajima Nei) and Neighbour joining analysis. Numbers above branches represent bootstrap percentages of 1000 replicates.

Percentage identity based on the 18S rRNA gene, was calculated using Kimura 2-parameter analysis between each of the groups. Within Groups 1-4, percentage similarity ranged from

93.5 to 97.4 % and between these groups, the average ranged from 87.8 to 92.7 % (Table 11.4). Group 1 showed the greatest level of difference to each of the other groups.

	Group 1	**Group 2**	**Group 3**	**Group 4**
Group 1	93.5			
Group 2	89.1	95.1		
Group 3	88.7	91.6	95.9	
Group 4	87.8	91.2	92.7	97.4

Table 11.4

Average percentage similarity of the 18S rRNA gene among and between groups using Kimura 2-parameter distance method (MEGA).

Percentage identity was also calculated for each of the subgroups (Table 11.5). Within subgroups, identity ranged from 92.6 to 97.8 % and average between subgroups, ranged from 87.4 to 93.8 %.

	1a	**1b**	**2a**	**2b**	**2c**	**T. youngi**	**3**	**4**
1a	95.9							
1b	91.7	92.6						
2a	89.3	88.3	97.2					
2b	88.8	88.0	92.8	95.0				
2c	89.7	88.7	93.8	92.5	97.2			
T. youngi	88.5	87.5	92.5	90.8	92.7	n/a		
3	89.0	88.3	92.6	91.3	91.5	90.4	97.8	
4	88.1	87.4	91.7	90.5	91.2	90.8	91.8	97.4

Table 11.5

Average percentage similarity of the 18S rRNA gene among and between subgroups using Kimura 2-parameter distance method (MEGA).

Within Group 2, subgroup 2a (the *Cytauxzoon* related spp.) was most genetically different from all other subgroups, while subgroup 2b and *T. youngi* both showed a similar level of percentage identity to subgroup 2c.

11.4.2 ITS and 5.8S rRNA gene

Sequence alignment using the ITS 2 and 5.8S rRNA gene was not reliable due to the high variability of the ITS 2 region between species and was therefore not used for phylogenetic analysis. Analysis was therefore conducted using a 150 bp region of the 5.8S rRNA gene.

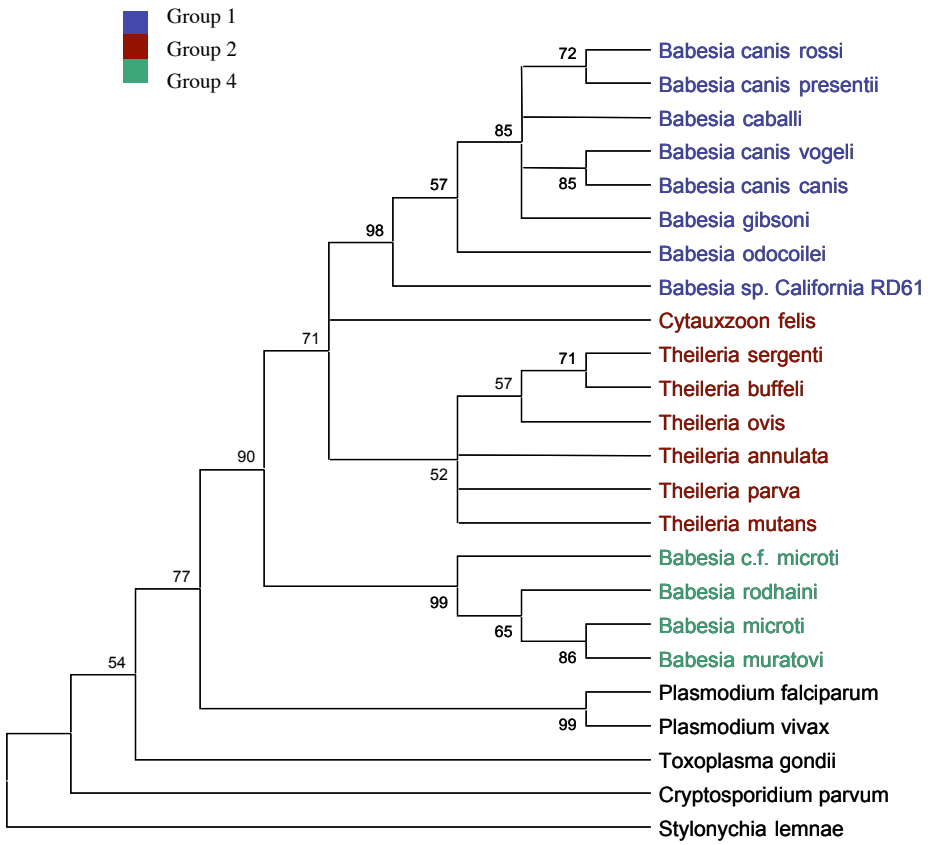


Figure 11.4

Phylogenetic tree constructed using 5.8S rRNA gene sequences based on Maximum Parsimony analysis. Numbers above branches represent bootstrap percentages of 1000 replicates

Both maximum parsimony (Figure 11.4) and distance (Figure 11.5) analysis produced three separate clades, consistent with Groups 1, 2 and 4 for the 18S rRNA analysis. *Cytauxzoon felis* formed an individual branch separate from both groups 1 and 2. Group 4, which included the *B. microti* related species was ancestral to both groups 1 and 2 with strong bootstrap support (90 %).

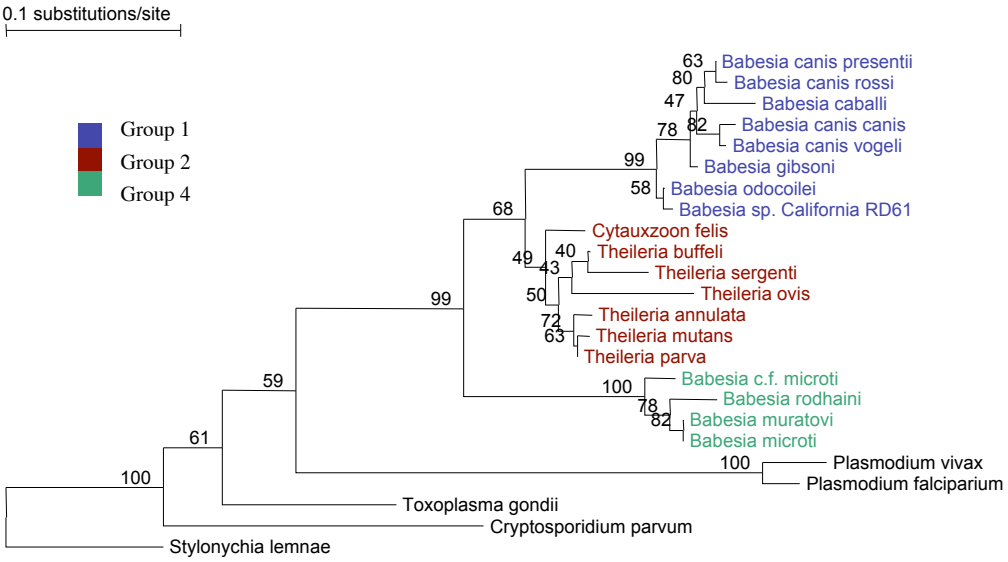


Figure 11.5

Phylogenetic tree constructed using 5.8S rRNA gene sequences based on distance (Tajima Nei) and Neighbour joining analysis. Numbers above branches represent bootstrap percentages of 1000 replicates.

The levels of genetic variation within and between each of the piroplasmid groups based on the 5.8S rRNA gene are shown in Table 11.6. Each group exhibited a similar level of homology both within (94.5 – 96.5 %) and between groups (73.5 – 84.9 %).

	Group 1	**Group 2**	**Group 3**
Group 1	96.4		
Group 2	84.9	94.4	
Group 3	73.5	80.0	96.5

Table 11.6

Average percentage similarity of a partial region of the 5.8S rRNA gene among and between subgroups using Kimura 2-parameter distance method (MEGA).

An additional analysis was conducted using a smaller region of the 5.8S rRNA gene (133 bp) in an effort to include additional species including the *B. duncani* (WA 1) group species.

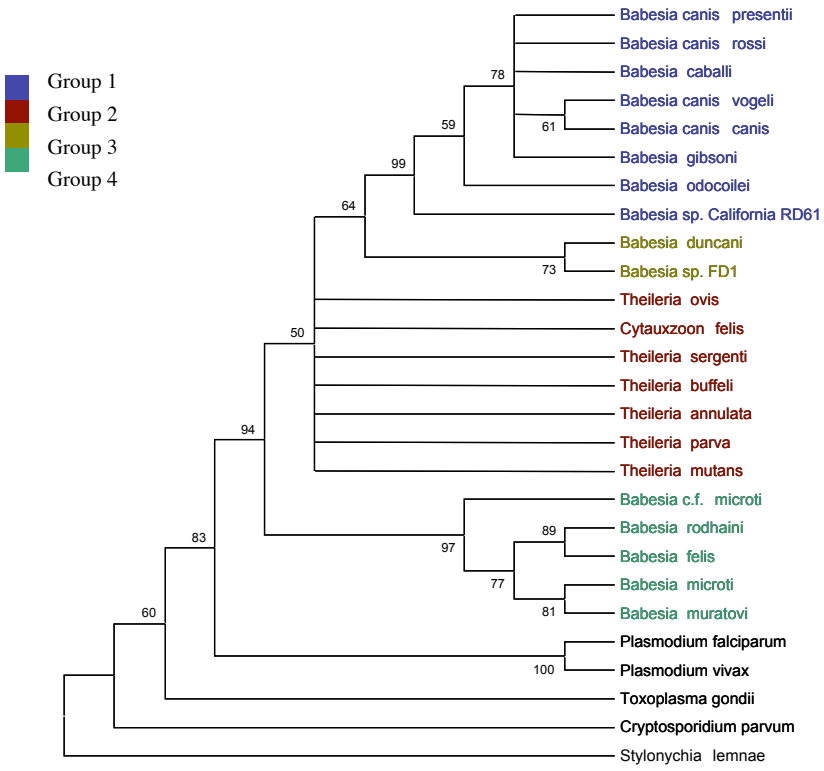


Figure 11.6

Phylogenetic tree constructed using partial 5.8S rRNA gene sequences based on Maximum Parsimony analysis. Numbers above branches represent bootstrap percentages of 1000 replicates.

While the *B. microti* group species (Group 4) were shown to be ancestral to all other piroplasm species with significant statistical support, no accurate resolution of the phylogenetic position of the remaining piroplasmids was produced using maximum parsimony (Figure 11.6).

Distance based analysis produced the separation of four distinct clades, similar to those produced using the 18S rRNA gene, however only three were supported by strong bootstrap values (Figure 11.7). *Babesia duncani* (WA 1) and *Babesia* sp. FD 1 formed a separate clade

(Group 3), but were grouped with the *Babesia* spp of Group 1, rather than the *Theileria* spp witnessed in the 18S rRNA distance analysis.

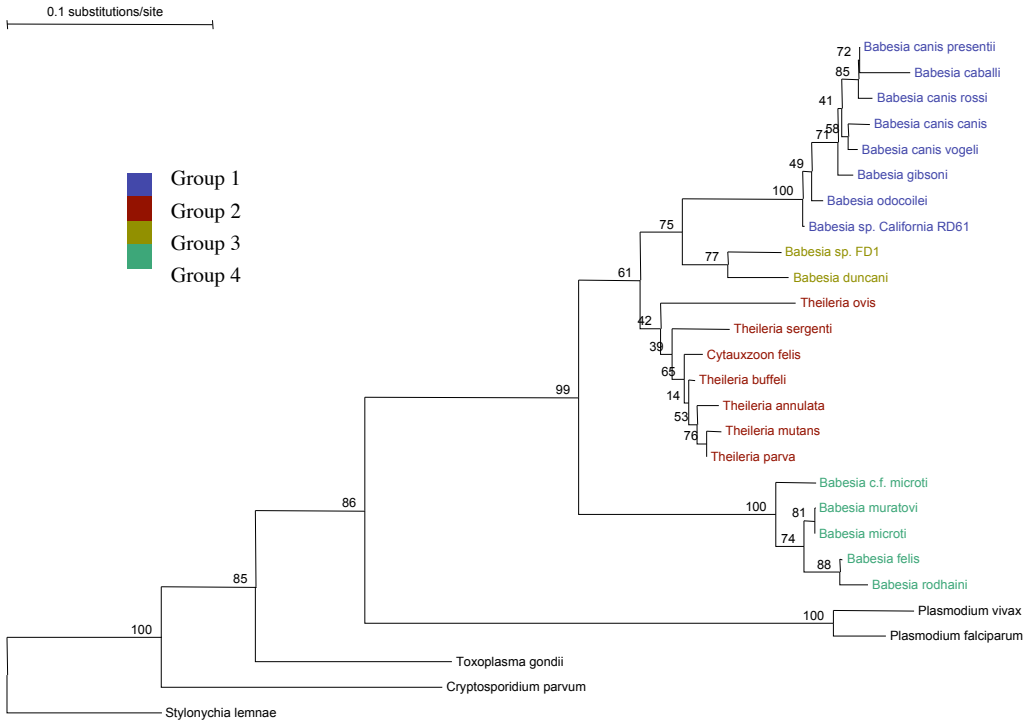


Figure 11.7

Phylogenetic tree constructed using 5.8S rRNA gene sequences based on distance (Tajima Nei) and Neighbour joining analysis. Numbers above branches represent bootstrap percentages of 1000 replicates.

11.4.3 Partial HSP 70 analysis

Phylogenetic trees produced using a partial region of the HSP 70 gene revealed three distinct clades, each with significant bootstrap support for both maximum parsimony (Figure 11.8) and distance (Figure 11.9) analysis. The *Babesia* spp were revealed as a paraphyletic taxa, forming two distinct groups (Group 1 and 4). Group 1 and 2 (containing the *Theileria* spp.) formed sister clades to one another and together with *Toxoplasma*, formed a separate clade from all other species. Group 4 was shown to be distinct from the other piroplasms and clustered with the remaining outgroup species, *Cryptosporidium*, *Eimeria* and *Plasmodium*.

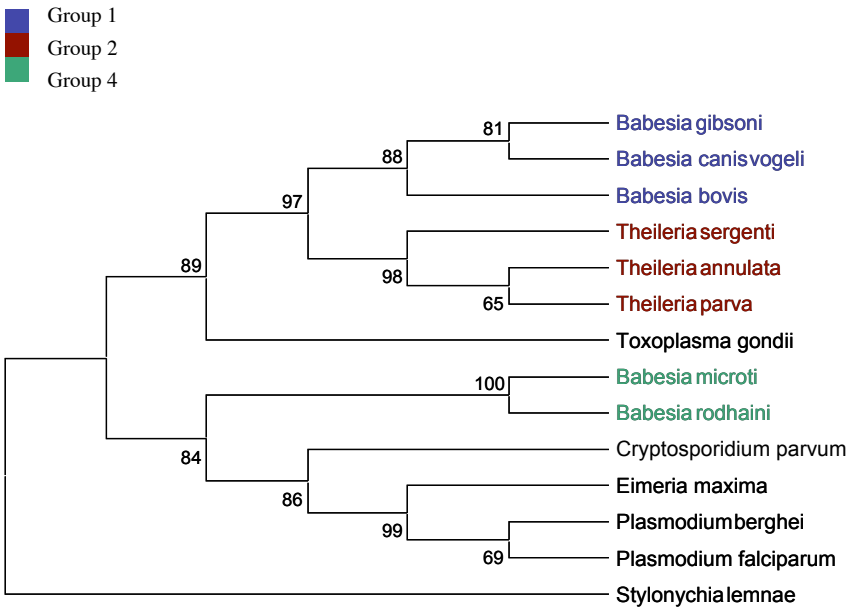


Figure 11.8

Phylogenetic tree constructed using partial HSP 70 gene sequences based on Maximum Parsimony analysis. Numbers above branches represent bootstrap percentages of 1000 replicates.

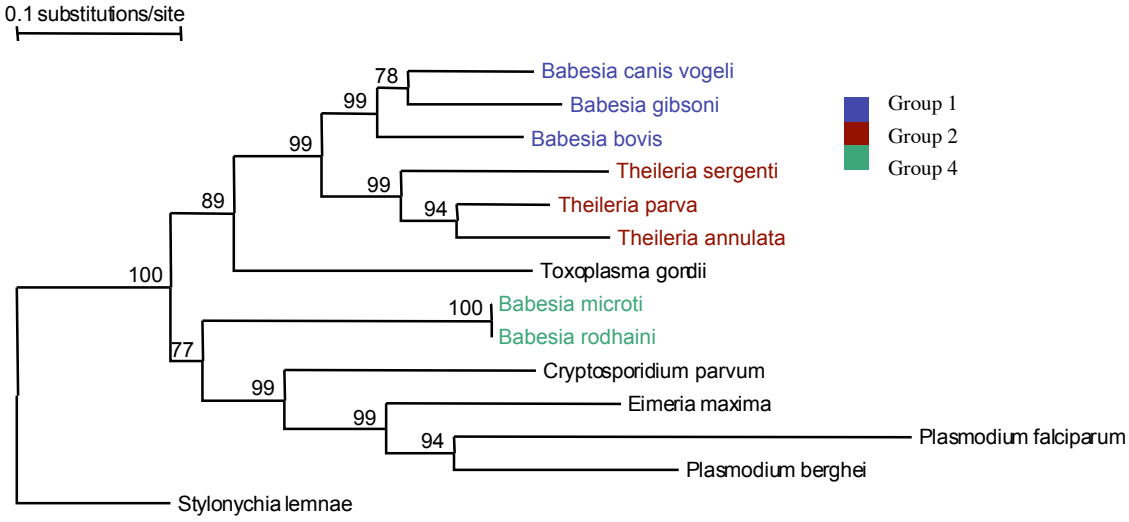


Figure 11.9

Phylogenetic tree constructed using HSP 70 gene sequences based on distance (Tajima Nei) and Neighbour joining analysis. Numbers above branches represent bootstrap percentages of 1000 replicates.

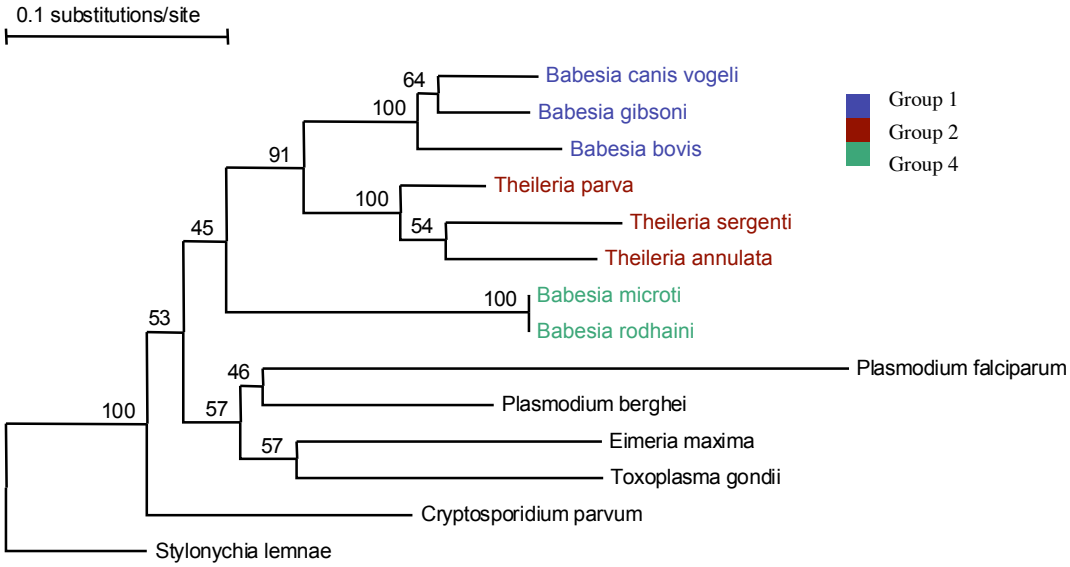


Figure 11.10

Phylogenetic tree constructed using partial HSP 70 gene sequences based on distance (Gaultier and Gouy) and Neighbour joining analysis. Numbers above branches represent bootstrap percentages of 1000 replicates.

Variation in the position of *B. microti* and *B. rodhaini* (Group 4) was seen using Tajima and Nei (Figure 11.9) and Gaultier and Gouy (Figure 11.10) based distance analyses. The Gaultier and Gouy tree clustered all piroplasmid spp. together in a clade separate to the outgroup spp., although bootstrap support for this placement was not significant.

	Group 4	**Group 1**	**Group 2**
Group 4	0.00		
Group 1	61.0	82.6	
Group 2	60.4	72.4	80.7

Table 11.7

Average percentage similarity of the partial HSP 70 gene among and between subgroups using Kimura 2-parameter distance method (MEGA).

Percentage identity values were calculated between Groups 1, 2 and 4 using the partial HSP 70 gene (Table 11.7) and revealed a similar level of homology between Group 4 and Groups 1 and 2.

11.4.4 Combined gene loci analysis

The combined 18S rRNA and HSP 70 gene analysis produced high support (98% bootstrap support using distance and 82% using maximum parsimony) for the grouping of all Piroplasmida species together in a clade separate to other apicomplexan species (Figures 11.11 and 11.12). The Piroplasmida were further dived into two major clades (each forming individual clades), one containing the *Theileria* (Group 2) and *Babesia* (Group 1), while the other contained *B. microti* and *B. rodhaini* (Group 4).

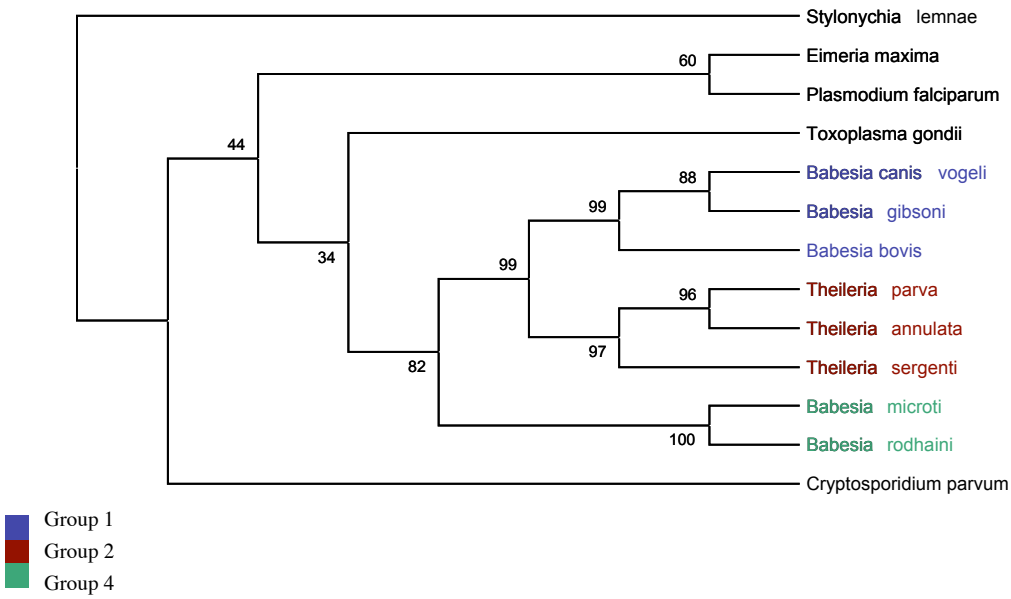


Figure 11.11

Phylogenetic tree constructed using partial 18S rRNA gene and HSP 70 sequences based on Maximum Parsimony analysis. Numbers above branches represent bootstrap percentages of 1000 replicates

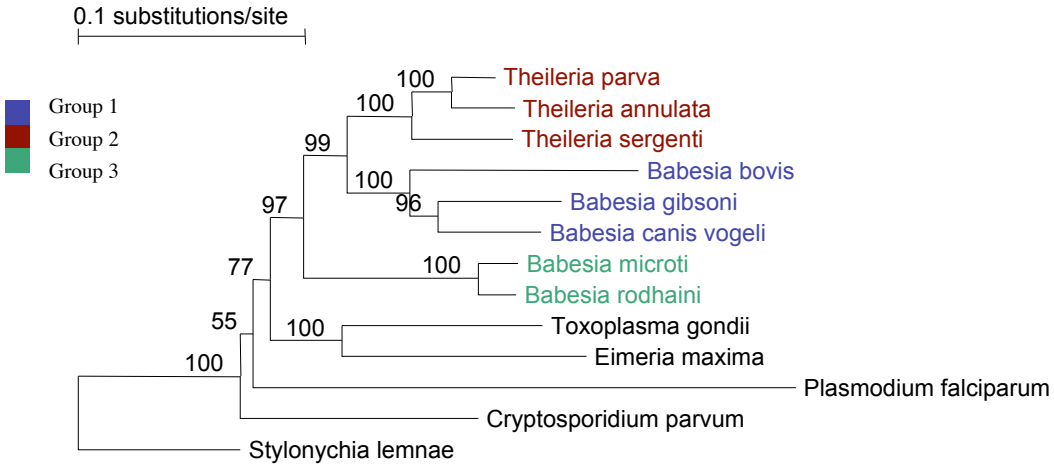


Figure 11.12

Phylogenetic tree constructed using partial 18S rRNA gene and HSP 70 sequences based on distance (Tajima Nei) and Neighbour joining analysis. Numbers above branches represent bootstrap percentages of 1000 replicates.

The combined loci phylogenetic trees can also be compared to the 18S rRNA analysis of the same selected species. Maximum parsimony analysis produced an identical grouping of the piroplasmid species (Figure 11.13), while distance based analysis placed *B. microti* and *B. rodhaini* in a clade with the *Theileria* (Figure 11.14).

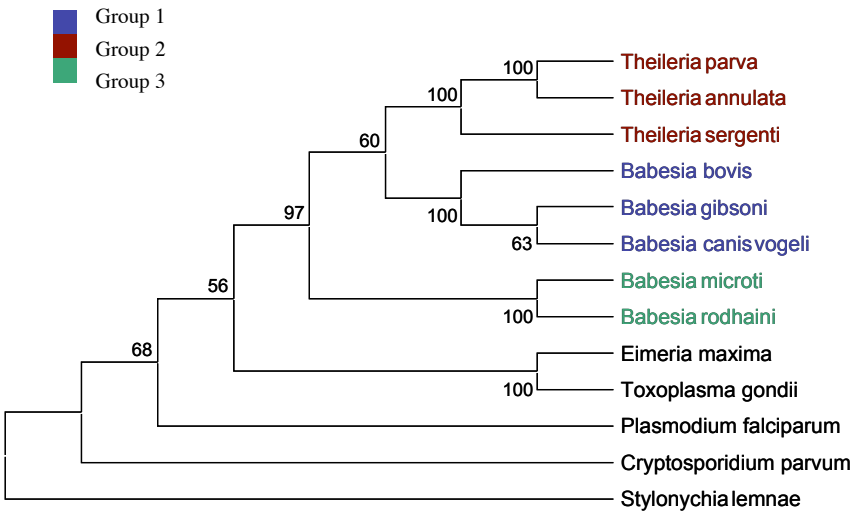


Figure 11.13

Phylogenetic tree constructed using partial 18S rRNA gene sequences based on Maximum Parsimony analysis. Numbers above branches represent bootstrap percentages of 1000 replicates.

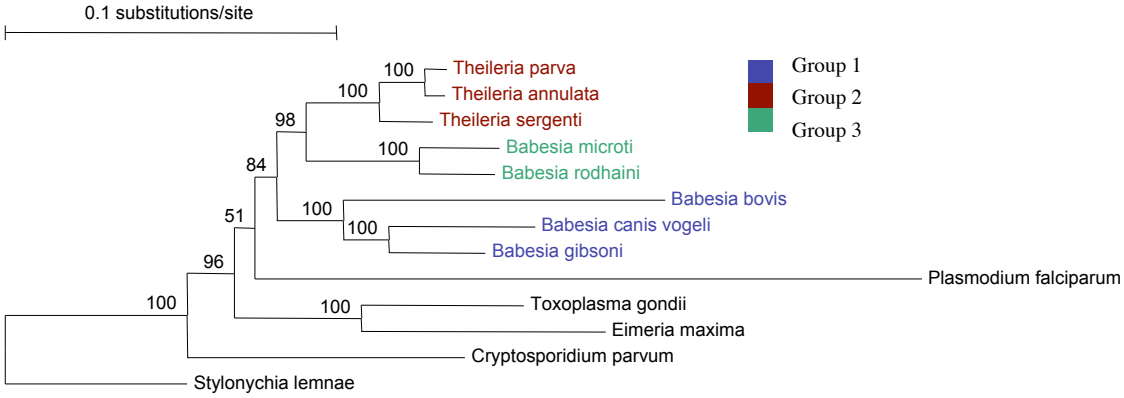


Figure 11.14

Phylogenetic tree constructed using partial 18S rRNA gene sequences based on Maximum Parsimony analysis. Numbers above branches represent bootstrap percentages of 1000 replicates.

11.5 Discussion

11.5.1 Phylogenetic relationships among the Piroplasmida

For the first reported time, this study has evaluated the evolutionary relationships of the Piroplasmida using a multi-gene approach and has thus allowed for a greater level of insight into the complicated phylogeny of the piroplasmids. While the use of a single gene locus may accurately reflect the evolution of that gene, though it may not be a true reflection of the overall evolution of the organism/s under study. This concern has been repeatedly voiced for many groups of organisms and a multi-gene approach has been suggested as reflecting more accurate evolutionary relationships (Devulder *et al*., 2005; Hypsa *et al*., 2005). Although the use of multiple gene loci may increase the accuracy of phylogenetic analyses conducted, two significant limitations hinder the widespread application of such methodology. These are the high number of partial gene sequences available on the GenBank database, and the lack of multiple loci being sequenced for most of the piroplasmid species. While only a limited group of species were used in the combined gene loci analysis, more accurate phylogenetic positioning will be possible as more species are sequenced on the basis of multiple genes. Indeed, a definitive understanding of the evolutionary relationships between most groups of organisms may not be accurately determined until full genomes are sequenced, seen already for *T. parva* (Gardner *et al*., 2005) and *T. annulata* (Pain *et al*., 2005).

While previous studies have divided the piroplasmids into five major groups on the basis of the 18S rRNA gene (Criado-Fornelio *et al*., 2003b; Reichard *et al*., 2005), this study concentrated on a more conservative division of four distinct groups of piroplasmids, a categorization also suggested by Penzhorn *et al*. (2001) and Dantrakool *et al*. (2004). The most ancestral groups, based on the 18S rRNA gene, were Groups 3 and 4.

Group 4 is equivalent to the Archeopiroplasmids described by Criado-Fornelio *et al*. (2003) and are piroplasm species that are considered ancestral to both the *Theileria* and *Babesia* spp. A notable feature in the phylogenetic analysis of Group 4 was that, occasionally,

particularly when using distance-based analysis of the 18S rRNA gene, this group was shown only to be ancestral to the *Theileria* spp. of Group 2 and not Group 1. Such placement of this group has also been replicated in other studies (Penzhorn *et al.*, 2001) but is likely to be misleading, a feature pointed out by Criado-Fornelio *et al*. (2003b) who suggested that using the substitution rate calibration method (Van de Peer and De Wachter, 1996) allows for a more accurate tree topology to be generated. The choice of outgroup species may also influence accurate phylogenetic positioning and further investigation into the relationship between the Piroplasmida and other apicomplexans is necessary (refer to 11.5.2).

Further verification that this group (Group 4) of piroplasms are indeed ancestral to all other characterised species was provided by analysis of the 5.8S rRNA gene, HSP 70 gene and combined 18S rRNA and HSP loci. Interestingly, on the basis of analysis of the HSP 70 gene, Group four showed a greater affinity to other Apicomplexan species such as *Cryptosporidium*, *Eimeria* and *Plasmodium*, offering additional support for the primative nature of this group of piroplasmids.

Another feature of this Group 4 is that the most ancestral species, notably *B. rodhaini* and *B. leo* are both piroplasms from Africa, thereby agreeing with the theory that Africa is the possible origin of all piroplasmids (Penzhorn *et al.*, 2001; Criado-Fornelio *et al.*, 2003b). Species belonging to this group have so far only been described in three broad mammalian taxa, the Rodentia, the Primates and the Carnivora, but it is difficult to determine whether a correlation between host and piroplasm evolution exists due to the limited number of species included in this analysis. Interestingly, the Rodentia and Primates are considered sister taxa within mammalian evolution (Jow *et al.*, 2002; Reyes *et al.*, 2004) and could provide a possible link within this group of piroplasms and explain the ability of *B. microti* to infect both rodents and humans, further raising questions about the zoonotic potential of other species within this group, such as *B. felis, B. leo* and *T. annae*. Limited information is

available on the tick vectors of the members of this group, preventing speculation into any evolutionary relationship between these and their respective hosts.

Group 3 corresponds to the Western USA *Babesia* clade described by Kjemtrup *et al.* (2000b) and later referred to as the Prototheilerids by Criado-Fornelio *et al.* (2003b). Unfortunately, no further support for the phylogenetic position of this group produced by 18S rRNA gene analysis was possible on the basis of other gene loci, due to only small sequence fragments being available for the 5.8S rRNA gene and the absence of any other published gene sequence data for this group. Further research into the molecular characterisation of species of this group using multiple gene loci needs to be investigated to clarify its position.

Group 1 and 2 were generally confirmed to be sister taxa using multiple analyses in this study. Group 1 should be considered homologous to the Babesids and Unguilibabesids described by Criado-Fornelio *et al.* (2003b) and has been suggested to represent the genus *Babesia – sensu stricto* (Reichard *et al.*, 2005). The species within this group are the most recently evolved of all the piroplasmids. Group 2 is consistent with the Theilerids described by Criado-Fornelio *et al.* (2003b). The most ancestral species within this group were the *Cytauxzoon* species, the *T. equi* like species, *B. bicornis* and *T. youngi*. Early investigations suggest that the marsupial piroplasms are ancestors to the Theileriidae and closely related to the *Theileria equi* and *Cytauxzoon* spp groups (Lee, 2004) and may provide greater insight into the evolution of this group.

It is important to understand that a definitive theory on the evolution of the Piroplasmida is difficult to achieve without the inclusion of key piroplasm species from fish, amphibians, reptiles, birds, marsupials and other mammals. The majority of species that have been molecularly characterised are of veterinary and medical significance, with regrettable neglect

of the wildlife piroplasms. No species described by Levine (1988) as belonging to the family Haemohormidiiae, which include piroplasms of fish, amphibia, reptiles and birds or the family Anthemosomatidae of mammals have been molecularly characterised and therefore future study to investigate these species is pertinent to this discussion of phylogeny. While lineages of piroplasm species are likely to follow the evolutionary patterns of their vertebrate hosts, the role of invertebrate hosts should not be underestimated. Piroplasm species may also follow the evolutionary patterns of ticks. This may include decreased host specificity of some tick species that results in a rapid change in piroplasm hosts, for example a bird tick that evolves to also parasitize mammalian hosts. Thus, the evolution of the piroplasmids becomes increasingly complicated and difficult to define and conclusions based solely on current host-parasite relationships may not be sufficiently robust.

11.5.2 Phylogenetic relationship of the Piroplasmida to other Apicomplexan Taxa

This study has also shown that on the basis of the 18S rRNA gene, HSP 70 gene and combined loci, the relationship of the Piroplasmida to other Apicomplexan taxa is somewhat inconclusive and is likely to be a reflection of the small number of Coccidian and other Apicomplexan species analysed. Historically, both the Piroplasmida and Haemosporida, which include the genera *Plasmodium*, *Hepatocystis*, *Haemoproteus* and *Leucocytozoon* have been considered to be sister orders (together forming the Haematozoa) as both groups of protozoa have similar life cycle stages, including the existence of an arthropod vector stage and an intraerythrocytic stage within the vertebrate host (Levine, 1988). Early phylogenetic analysis of species from each of the two orders on the basis of the 18S rRNA gene was inconclusive, as the *Theileria* and *Babesia* species were not statistically more closely grouped with *Plasmodium* than the coccidia *Toxoplasma, Neospora* and *Sarcocytis* (Escalante and Ayala, 1995). Other studies, also using the 18S rRNA gene have suggested that the coccidians are indeed ancestral to both the Piroplasmida and the Haemosporida (Van de Peer and De Wachter, 1997; Dantrakool *et al.*, 2004). The uncertain phylogenetic relationships between the Piroplasmida and Haemosporida is also reflected in analyses of the

HSP 90 gene (Stechmann and Cavalier-Smith, 2003). Further analysis of multiple gene loci from a large number of species is needed to better understand the evolutionary relationships among the apicomplexan taxa and may also allow for a more accurate view of the phylogeny of the Piroplasmida.

11.5.3 Taxonomic relationships among the Piroplasmida

This study has shown that phylogenetic analysis of the Piroplasmida using the 18S rRNA, 5.8S rRNA and HSP 70 genes exposes the current paraphyly that exists among multiple taxa, with members of the genus *Babesia* being located within three separate clades and the *Theileria* belonging to two separate groups. This is a concept supported by a number of previous investigations (Allsopp *et al.*, 1994; Zahler *et al.*, 2000a; Ruef *et al.*, 2000; Reichard *et al.*, 2005).

A re-occurring problem with the piroplasmids is the difficulty in accurately assigning a newly described species to a genus and combined with complex historical classification systems, has resulted in both the genera *Babesia* and *Theileria* becoming paraphyletic taxa. There is consequently an overwhelming need to verify the taxonomic status of multiple new species of piroplasm before they are named. Careless assignment of new species of piroplasm to incorrect genera and the continual description of new species with simple code names, adds to the ambivalent nature of the current taxonomic scheme for the order Piroplasmida. The practicality of continually referring to a species as for example, WA 1, BH 1 or *Cytauxzoon* sp. (Iberian Lynx) is limited and especially confusing in the latter case if more than one piroplasm spp. infect the described host. A more useful description of new species, following the International Code of Zoological Nomenclature (Ride *et al.*, 1999) is suggested, including verification of the genus on the basis of two or more phylogenetically informative gene loci. Perhaps the most significant problem with describing a new species on the sole basis of molecular characterisation is the possibility that this species has already previously been described using phenotypic characteristics during the pre-molecular

biological era. It is impossible to ascertain whether this is indeed the case, without any of the originally described protozoa being available for subsequent molecular analysis.

A means of alleviating the current taxonomic discordance within the order Piroplasmida is by establishing three families and re-categorising all species within five genera. It is difficult to definitively define a family level classification, an artificial construct designed purely for convenience, although molecular based analysis may offer a solution. Previous studies that have determined the taxonomic status of selected piroplasmids using molecular analysis have proposed percentage identity as the basis of discimination (Schnittiger *et al.*, 2003) and is also a feature used for species level separation (discussed in Chapter ten).

Groups 1 and 2 are consistently recognised as separate groups in this study and in multiple other analyses (Penzhorn *et al.*, 2001; Reichard *et al.*, 2005). It is also postulated to correspond to the families the Babesiidae (Group 1) and the Theileriidae (Group 2). All other groups can therefore be defined as a family or not based on the mean percentage identity comparison with these two established families. Group 1 displayed a similar average percentage identity using the 18S rRNA between Groups 2, 3 and 4. Likewise, the average percentage identity between Group 2 and Groups 3 and 4 were also similar. This was a feature that also existed between Groups 1, 2 and 4 based on the 5.8S rRNA and HSP 70 genes. The phylogenetic separation and the percentage identity using multiple gene loci therefore provides the basis of the proposed taxonomic changes to each group of piroplasmid and is discussed in section 10.5.5.

10.5.4 Limitations of phenotypic characters as the basis for taxonomic classification

It has been argued that molecular-based taxonomy can result in the over-zealous creation of new taxa, often at the expense of many decades of classification using traditional criteria (Uilenberg *et al.*, 2004). Preference to the use of combined genotypic and phenotypic characters is therefore suggested, however traditional characters used to define the

Piroplasmida currently show significant limitations by disagreeing with the results of phylogenetic analyses and failing to allow for the accurate taxonomic classification of this group of protozoa. The separation of the piroplasmids into three or possibly four families is not supported at present by any phenotypic characteristics due to the following reasons:

i) Morphology

The morphological similarity of all the piroplasm species makes it difficult to distinguish each of the proposed family groups on the basis of phenotype. Both 'small' (typically 1-3 μm in diameter) and 'large' (3-5 μm) species of piroplasm are known to exist. All large piroplasms reported to date are confined to the Babesiidae, yet small-type species also exist within this family, such as the small *B. gibsoni*. In each of the three remaining families, only small morphological species have been reported. Intracellular organelles have also been used to distinguish families within the Piroplasmida with the Babesiidae reported to have an apical complex reduced to a polar ring and the presence of rhoptries and subpellicular microtubules (Levine, 1988). The Theileriidae have reduced elements of the apical complex, always include rhoptries, are without polar ring or conoid and usually are without subpellicular microtubules (Levine, 1988). It is important to note that such detailed morphological descriptions of many piroplasm spp are absent or incomplete. Further investigation of the morphology of members of the Piroplasmida is therefore necessary to determine whether possible phenotypic differences exist between each Family group.

ii) Number of merozoites

A traditional characteristic used to separate certain taxa within the Piroplasmida is the number of dividing merozoites formed within a single erythrocyte of the vertebrate host. Members of the family Anthemosomatidae are reported to form between five and 32 merozoites within the host erythrocyte, while members of all other piroplasm families are suggested to produce two to four merozoites (Levine, 1981; Levine, 1988). Recent investigations have shown that five merozoites are observed in infections of a new *Babesia*

sp. of bandicoot rats in Thailand (Dantrakool *et al*., 2004) and in severe combined immune deficiency (SCID) mice, as many as 32 merozoites of *B. gibsoni* were observed in a single erythrocyte (Fukumoto *et al*., 2000).

Previous studies have also speculated that the Theileridae may be differentiated from the Babesiidae on the basis of merozoite morphology. It was suggested that only *Theileria* spp developed a tetrad of dividing merozoites, which produced a 'maltese cross' formation. The discovery of maltese cross forms of *B. microti*, then lead to speculation that these piroplasms were possibly more closely related to the Theileriidae (Zahler *et al*., 2000a). Merozoites in a maltese cross formation have however now been reported in multiple species. Within Group 4, *B. microti* (Yokoyama *et al*., 2003), *B. leo* (Penzhorn *et al*., 2001) and *Entopolypoides macaci* (Bronsdon *et al*., 1999). Within the Group 3, the maltese cross formation has been observed for *Babesia* sp. WA 1 (Thomford *et al*., 1993) and within the Theileriidae, *T. parva* (Fawcett *et al*., 1987) and *T. equi* (Mehlhorn and Schein, 1998). A recent report has also described maltese cross forms of *Babesia kiwiensis* (Pierce *et al*., 2003), a probable member of the Babesiidae (Down, 2004).

iii) Lifecycle characteristics

Very few detailed studies of the lifecycles of individual piroplasm spp have been published, making it difficult to correlate any characteristics between family groups with any level of assurity. The presence or absence of an exoerythrocytic stage has traditionally been a key characteristic used in defining the Theileriidae, which show invasion of the lymphocytes before intraerythrocytic development (Shaw *et al*., 2003) and the Babesiidae that develop solely within the erythrocytes of the vertebrate host (Mehlhorn and Schein, 1984). An exoerythrocytic stage is suspected to exist for *B. microti* (Homer *et al*., 2000) requiring further investigation. *Theileria buffeli* and *T. sergenti* have also been suggested to belong to an evolutionary lineage of non-lymphoproliferative *Theileria* spp. (Schnittger *et al*., 2000). Criado-Fornelio *et al*., (2003b) suggested that invasion of lymphocytic cells is a primative

characteristic of the piroplamids, a feature lost in the *Babesia* spp. as they became more specialised and cell specific.

iv) Host species

Defining families and genera among the piroplasmids on the basis of their vertebrate host is also futile, as a number of host species have now been reported to potentially become infected with multiple piroplasm species. For example, *B. canis presentii, B. canis canis, C. felis* and *B. felis* in cats (Criado-Fornelio *et al.*, 2003a; Baneth *et al.*, 2004; Reichard *et al.*, 2005), *B. bigemina, B. bovis, T. buffeli, T. mutans* and *T. velifera* in cattle and eight different piroplasm species have been found to infect dogs. Many piroplasm species are also capable of infecting multiple host spp., a feature that is increasingly being reported, such as *T. equi* infecting both horses and dogs (Criado-Fornelio *et al.*, 2003a), *B. divergens* infecting rabbits, rats, humans, sheep and cattle (Chauvin *et al.*, 2002; Goethert and Telford, 2003; Musa and Abdel Gawad, 2004) and *B. microti* being capable of infecting humans and rodents (Goethert and Telford, 2003).

11.5.4 Proposed taxonomic changes to the Order Piroplasmida

The following section describes two separate ways to attempt to resolve the current paraphyly and taxonomic confusion that exist in recent phylogenetic analyses and schemes of systematic classification. Both are tentative approaches and by no means can offer definitive solutions to the taxonomy of the order Piroplasmida, especially in light of the use of partial gene sequences and absence of gene sequences for many piroplasm species in the phylogenetic analyses within this chapter.

a) Proposal One: The re-organization of the Families Babesiidae and Theileriidae and establishment of the new Family Piroplasmiidae and resurrection of the genera Piroplasma *(Patton, 1895) and* Achromaticus *(Dionisi, 1899)*

- Family Babesiidae (Group 1 in this study)
- Genus *Babesia* - type species is *B. bovis* (Babes, 1888)

The Babesiidae comprises one of the most genetically variable and most recently evolved groups of piroplasms. They have been described in multiple mammalian and possibly bird host species, with the probable inclusion of *Babesia kiwiensis* upon further phylogenetic analysis (Down, 2004). Two distinct subgroups were found to separate the Babesiidae, homologous to the Babesids and Ungulibabesids clades proposed by Criado-Fornelio *et al.* (2003b). It is suggested however, that both of these descriptions by Criado-Fornelio *et al.* (2003b) are misleading due to the existence of ungulate species of *Babesia* in both groups, a view also supported by Reichard *et al.* (2005). Also in light of new species sequence data, the Ungulibabesid group also includes a species from a dog (Birkenheuer *et al.*, 2004b). While the existence of subgroups should be recognized, the taxonomic significance of these separations remains uncertain. All the Babesiidae should therefore remain classified within a single genus, until the inclusion of additional species and/or genetic analysis allows for further clarification of these subgroups.

- Family Theileriidae (Group 2 in this study)
- Genus *Theileria* – type species is *T. parva* (Theiler, 1904),
- Genus *Cytauxzoon* – type species proposed as *C. felis*.

The Theileriidae also represents a diverse group of piroplasmids, with the possible need for the sub-categorisation of this family into multiple genera to accurately reflect evolutionary relationships. The *Cytauxzoon* spp., with the possible inclusion of *T. bicornis* need to be formally described under the genus *Cytauxzoon*. This genus has previously been considered a synonym of the genus *Theileria* (Levine, 1988) and therefore must be redefined to avoid

confusion. It may also be deemed necessary to elevate the *T. equi* clade (Group 2b) to a genus level of classification, however as a similar level of genetic difference is seen in *T. youngi* when compared to other *Theileria* groups, it is suggested that these species remain within the genus *Theileria* until further analysis suggests otherwise. This includes reclassifying *B. bicornis* within the genus *Theileria* and the additional allocation of a new species name due to the pre-existence of the distinct species *T. bicornis* (Nijhof *et al.*, 2003).

- Family Piroplasmiidae nov. Fam. (Group 4 in this study)
- *Genus Piroplasma* (Patton, 1895) - type species proposed as *Piroplasma microti* (Franca, 1910)

The family Piroplasmiidae is likely to represent the most ancestral of the piroplasmids described to date and hence was described by Criado-Fornelio *et al.* (2003b) as the Archeopiroplasmids. This group of piroplasm species currently exists under a paraphyletic taxonomic system and is comprised of two genera, both *Theileria* and *Babesia*. While the need for the separation of this group of piroplasmids into a new genus or even family has been speculated before (Zahler *et al.*, 2000a), no definitive taxonomic solution has been postulated and to further add to the confusion, many species within this group have had multiple name changes in the past.

As an example, *Babesia microti* (Franca, 1912) was originally described as *Nicollia microti* (Franca, 1910) and this genus was also later suggested for *B. rodhaini* due to its phylogenetic separation from other *Babesia* and *Theileria* species (Ellis *et al.*, 1992). The family Nicolliidae was then first proposed by Allsopp *et al.* (1994) for the species *B. rodhaini*, *B. equi* and *C. felis* as a result of the suggested reclassification of *B. equi* to *Nicollia equi* (Krylov, 1981). *Babesia equi* was later re-classified as *Theileria equi* (Mehlhorn and Schein, 1998) and all subsequent genetic studies have found that both *T. equi* and *C. felis* show a closer affinity to the Theileriidae (Kjemtrup *et al.*, 2000b; Penzhorn *et al.*, 2001; Criado-Fornelio *et al.*, 2003b; Reichard *et al.*, 2005). Zahler *et al.* (2000a) also supported the notion

that *B. microti* and related piroplasm species should be classified under a third taxonomic entity at the family level of classification, a feature later supported by Criado-Fornelio *et al.* (2003b). To complicate matters further, the genus *Nicollia* has also been used to describe a Trychostrongilid nematode (Durette-Desset and Cassone, 1983).

A more suitable means of renaming this group is by selecting the oldest synonym[7] of the genus *Babesia*, not currently in use according to the ICZN (Ride *et al.*, 1999). It is therefore proposed that *B. microti, T. annae, B. rodhaini, B. leo*, *B. felis* and unnamed species/isolates *Babesia* sp. IoRK/HM101 (Saito-Ito *et al.*, 2004) and *Babesia* sp. Caracal be re-classified under the genus *Piroplasma* (Patton, 1895) in the newly proposed family, Piroplasmiidae, with the possible inclusion of the Baboon piroplasm *Entopolypoides macaci* (Bronsdon *et al.*, 1999) and *B. microti*-like isolates from raccoons (Goethert and Telford, 2003; Kawabuchi *et al.*, 2005), foxes (Goethert and Telford, 2003; Criado-Fornelio *et al.*, 2003a), skunks and humans (Goethert and Telford, 2003) upon further phylogenetic analysis.

- Family *incertae sedis* (possible creation of the Achromaticiidae nov. fam.)
- Genus *Achromaticus* (Dionisi, 1899) – type species proposed as *Achromaticus duncani* (formerly *Babesia* sp. WA1, *Babesia duncani*)

This group of piroplasmid species is somewhat problematic in both phylogenetic and taxonomic schemes of classification. The main reason for this is the limited biological and genetic studies conducted on these species and as a consequence it is difficult to allocate this group to an already established taxonomic entity or postulate that a new level of classification may be necessary. It has recently been suggested that the species commonly referred to as WA1 and the small canine piroplasm described in California should be classified within the genus *Babesia* as *B. duncani* and *B. conradae* (Kjemtrup *et al.*, 2005) respectively, yet is a feature disputed by the analyses described in this chapter.

[7] Article 60 of the International Code of Zoological Nomenclature. 60.2. **Junior homonyms with synonyms.** If the rejected junior homonym has one or more available and potentially valid synonyms, the oldest of these becomes the valid name of the taxon [Art. 23.3.5] with its own authorship and date.

While less genetic information is available for the WA1 related species, the 18S rRNA gene based identity between this group and the Babesiidae (88.7%) and the Theileriidae (91.6%) is similar to average identity shown between the Piroplasmiidae and these two families (87.8% and 91.2% resepectively). As only one gene could be accurately analysed, it is speculated that this group may represent a separate piroplasmid Family, but further research needs to be conducted to produce additional support for this idea. As a way of defining the phylogenetic separation of this group, a new genus is proposed in accordance to guidelines established by the ICZN, without the definitive inclusion of this group within an established family. The species, *Babesia* sp. WA1 (*Babesia duncani*), *Babesia conradae*, Piroplasmida gen. sp. FD1, CA1, CA2 and BH1 should each be reclassified under the genus *Achromaticus* (Dionisi, 1899), the third oldest synonym for the genus *Babesia*. The second oldest synonym is *Haematococcus* (Babes, 1889), however this genus is already established as a genus of algae (Hepperle *et al*., 1998) and therefore should not be considered for this group of piroplasmids. Interestingly, Uilenberg (1967) previously gave priority to the genus *Achromaticus* to describe members of the Babesiidae that formed tetrads, a feature observed for the species WA1 (Thomford *et al*., 1993).

Family	Current classification*	Proposed Classification			
		18S rRNA	5.8S rRNA	HSP 70	Taxonomic changes
Babesiidae	*Babesia bicornis*	*B. bigemina*			
	Babesia bigemina	*B. bovis*		*B. bovis*	
	Babesia bovis	*B. caballi*	*B. caballi*		
	Babesia caballi	*B. canis canis*	*B. canis canis*	*B. c. canis*	*Babesia canis*
	Babesia canis canis	*B. canis presentii*	*B. canis presentii*		*Babesia presentii*
	Babesia canis presentii	*B. canis vogeli*	*B. canis vogeli*	*B. c. vogeli*	*Babesia vogeli*
	Babesia canis vogeli	*B. canis rossi*		*B. c. rossi*	*Babesia rossi*
	Babesia canis rossi	*B. divergens*			
	*Babesia conradae**	*B. gibsoni*	*B. gibsoni*	*B. gibsoni*	
	Babesia divergens	*B. odecoilei*	*B. odocoilei*		
	*Babesia duncani**	*B. ovata*			
	Babesia felis	*B. ovis*			
	Babesia gibsoni	*B. venatorum* (EU1)			
	Babesia leo				
	Babesia microti				
	Babesia muratovi				
	Babesia odecoilei				
	Babesia ovata				
	Babesia ovis				
	Babesia rodhaini				
	Babesia venatorum EU1				
Theileriidae	*Theileria annae*	*T. annulata*	*T. annulata*	*T. annulata*	
	Theileria annulata	*T. bicornis*			*Cytauxzoon bicornis?*
	Theileria bicornis	*T. buffeli*	*T. buffeli*		
	Theileria buffeli	*T. cervi*			
	Theileria cervi	*T. lestoquardi*			
	Theileria lestoquardi	*T. mutans*	*T. mutans*		
	Theileria mutans	*T. ovis*	*T. ovis*	*T. parva*	
	Theileria ovis	*T. parva*	*T. parva*		
	Theileria parva	*T. sergenti*	*T. sergenti*	*T. sergenti*	
	Theileria separata	*T. separata*			
	Theileria sergenti	*T. taurotragi*			
	Theileria taurotragi	*T. velifera*			
	Theileria velifera	*T. youngi*			
	Theileria youngi	*Cytauxzoon felis*	*C. felis*		
	Cytauxzoon felis	*Cytauxzoon manul*			
	Cytauxzoon manul	*Babesia bicornis*			*Theileria* sp.
Piroplasmiidae		*Theileria annae*			*Piroplasma annae*
		Babesia felis	*B. felis*		*Piroplasma felis*
		Babesia leo			*Piroplasma leo*
		Babesia microti	*B. microti*	*B. microti*	*Piroplasma microti*
		Babesia rodhaini	*B. rodhaini*	*B. rodhaini*	*Piroplasma rodhaini*
			B. muratovi		*Piroplasma muratovi*
Uncertain	Piroplasmida sp. BH1	BH1			*Achromaticus* sp.
	Piroplasmida sp. FD1	FD1	FD1		*Achromaticus* sp.
		Babesia conradae			*Achromaticus conradae*
		Babesia duncani	*B. duncani*		*Achromaticus duncani*

Table 11.8

Proposed taxonomic changes to the order Piroplasmida (*most un-named species have been omitted)

b) Proposal Two: Reorganization of all species of order Piroplasmida into two families; the Theileriidae and the Babesiidae.

The second option for a proposal for changes to the taxonomic classification of the order Piroplasmida offers a more simplified and less ambiguous approach by dividing the piroplasms into just two separate family groups. Support for the proposed taxonomy is provided by both phylogenetic and traditional biological characteristics.

- Family Babesiidae (Group 1 in this study)
- Genus *Babesia* - type species is *B. bovis* (Babes, 1888)

Members of the family Babesiidae and genus *Babesia* under the second proposed scheme of classification is identical to that described under the first proposal.

- Family Theileriidae (Groups 2, 3 and 4 in this study)
- Genus *Theileria* – type specimen is *T. parva* (Theiler, 1904),

The second proposal for the family Theileriidae suggests that all piroplasm species ancestral to the Babesiidae (groups 2, 3 and 4) are included in the one family and are all classified as belonging to the genus *Theileria* and the removal of the currently synonymous genus *Cytauxzoon*. This is perhaps the most practical solution to the current paraphyly that exists, yet may not be the most scientifically sound. Support for this proposal is provided by the similar morphology observed between each of the included piroplasm species, all are typically small (1-3 μm in diameter), are capable of producing a maltese cross lifecycle stage and the general existence of a lymphocyctic stage in most species. While both small size and the maltese cross formation are also observed in members of the Babesiidae, no large form species have been described for the Theileriiidae. These biological characteristics can be considered primitive in accordance with suggestions made by Criado-Fornelio *et al.* (2003b) and the ancestral phylogenetic position of these protozoa.

11.5.5 Conclusion

This chapter has provided strong evidence to support the increased application of molecular-based analysis to better understand the phylogenetic relationships, and clarify taxonomic discordance amongst the order Piroplasmida. This study has also highlighted the advantages of using multiple genes, including combined loci, to reflect the evolution of the selected organisms, rather than just of a selected gene. Further biological and genetic evidence is needed before definitive taxonomic changes can be made for this group of protozoa as suggested with both proposed schemes of classification in this chapter. It is anticipated that reclassifying the Piroplasmida into re-defined families and genera, will help to alleviate the current paraphyly and overall taxonomic confusion among this important group of apicomplexan parasites.

CHAPTER TWELVE

General Discussion

12.1 Emergent tick-borne pathogens in Australia and quarantine implications

This thesis has encompassed two emergent tick-borne diseases of dogs in Australia and has allowed for a significant increase in the current knowledge regarding the molecular epidemiology of these infections. Previous to this study, *B. gibsoni* had only been reported in three dogs in Victoria, eastern Australia (Muhlnickel *et al.*, 2002). Subsequently, infections of *B. gibsoni* have been described in many American Pit Bull Terriers between the localities of Warrnambool and Ballarat in Victoria (Chapter seven) and the first case of this infection in New South Wales has also been reported (Chapter six). A greater insight has also been provided into the transmission dynamics of enzootic *B. gibsoni* infections in Victoria and has suggested that blood-to-blood transmission occurring during dog fighting may be a significant factor in the spread of this disease (Chapter seven). Changes to the management practices of certain populations of American Pit Bull Terriers could be considered in an attempt to control the spread of this disease but as dog fighting is already an illegal activity in Australia, this is unlikely to be a feasible goal. The definitive role of tick vectors remains unknown and requires further investigation, as both *R. sanguineus* and *H. longicornis* are present within Australia (Hoogstraal *et a*l., 1968; Roberts, 1970). Additional research into an effective drug treatment for *B. gibsoni* is also paramount if this disease is to be controlled.

Highlighted by studies in this thesis, are the limitations that exist for each diagnostic method currently available for the detection of *B. gibsoni* and indeed that no 'gold standard' detection technique is available. Evaluation of the current screening protocol, which includes

detection using microscopic examination of a blood smear and IFAT, for dogs being imported into New Zealand, revealed the low sensitivity of microscopic detection. PCR-RFLP is suggested as a suitable replacement of microscopy. The use of both IFAT and PCR offer the greatest assurance currently available in accurately detecting *B. gibsoni* during all stages of infection, particularly by PCR during early stage infections and by IFAT during chronic stage infections. Increasing the cut-off titre of IFA testing for positive *B. gibsoni* infections from 1: 40 to 1 : 160 is also suggested as a means of reducing the number of false positive produced by antigen cross-reaction. The combination of different IFAT and PCR results for dogs being imported into New Zealand are each given in Table 12.1, along with possible explanations and recommendations for each result. Each recommendation is the culmination of results presented in Chapters five, six, seven and eight.

Test result	Explanation	Recommendation
i) PCR positive (*B. gibsoni*), IFAT positive (≥1:160)	Considered a true positive result, suggesting the presence of circulating *B. gibsoni* with the venous blood and the development of an immune response to the infection.	Should not be imported into New Zealand.
ii) PCR positive (other piroplasm spp), IFAT positive (≥1:160)	Antigen cross-reaction with another piroplasm spp, producing a high antibody titre. Not *B. gibsoni* positive.	If *B. canis vogeli*, may be allowed entry into New Zealand.
iii) PCR positive (*B. gibsoni*), IFAT negative (<1:160)	Considered a true positive result, suggesting the presence of circulating *B. gibsoni* within the venous blood and failure of the dog to seroconvert or infection is only at an early stage.	Should not be imported into New Zealand.
iv) PCR negative, IFAT positive (≥1:160)	May be a true positive result as the infection may be at a chronic stage and limited or a total absence of circulating *B. gibsoni* within the venous blood exists. This situation could also represent a false positive result produced by a non-specific antigen cross-reaction (eg – *Neospora caninum*)	Should not be imported into New Zealand.
v) PCR negative, IFAT negative (<1:160)	Considered a true negative result however, may arise if a dog fails to seroconvert and no *B. gibsoni* is found within the venous blood, such as in chronic infection	Allowed entry into New Zealand

Table 12.1

Defining levels of detection using PCR and IFAT and the New Zealand quarantine implications of each result.

This thesis also reports for the first time, the detection of *A. platys* in Western Australia, Queensland and Victoria (Chapter nine). It is probable that *A. platys* is widespread throughout Australia and could be a reflection of the wide distribution of *R. sanguineus*. Further study must however be conducted to verify this tick as the vector of *A. platys*. Also investigated for the first time was the pathogenesis of *A. platys* infection in dogs in northern Australia, the influence of co-infection with *B. canis vogeli* and the efficacy of doxycycline drug therapy. While studies conducted suggested that *A. platys* infection is somewhat benign, this could simply be a reflection of the immune status and previous exposure to this infection by the host. As there still remains reports of dogs developing signs of lethargy, fever and bleeding tendencies (Jefferies, 2001), additional research into dogs naive to *A. platys* exposure is necessary to further understand the pathogenesis of this infection. The role of sylvatic reservoirs in Australia, such as dingoes, feral dogs and foxes is poorly understood and may contribute to the transmission dynamics of both *A. platys* and the canine *Babesia* species. Further investigation into tick-borne disease of wild canine species should therefore also be considered.

Biosecurity is a pertinent issue in Australia and New Zealand, with both countries reportedly free from many significant pathogens of dogs such as rabies virus and *Ehrlichia canis* (Irwin, 2001; Mason *et al.*, 2001; Davidson, 2002). With increased levels of pet travel worldwide, the surveillance for exotic diseases in animals being imported and exported is of great importance. Both the recent discovery of *B. gibsoni* and *A. platys* in Australia and the limitations of current screening protocols for tick-borne diseases in dogs entering Australia and New Zealand, also expose the need to review current quaratine measures in an effort to prevent the importation and possible establishment of exotic pathogens into these countries. By implicating both serological and molecular-based detection methods for screening dogs, the risk of importing exotic tick-borne diseases including the highly pathogenic *B. canis rossi* and *E. canis*, can be minimized and should be considered by quarantine authorities in the future.

12.2 Molecular phylogeny and taxonomy of the Piroplasmida

This thesis has described the molecular characterisation of canine piroplasm isolates from many countries for the first time, giving a greater insight into the levels of molecular variation and worldwide distribution of the canine *Babesia* species, including the possible existence of genotypes associated with separate geographic locations. DNA sequencing of the HSP 70 for *B. canis vogeli* and the ITS 1, 5.8S, ITS 2 loci for *B. gibsoni* has also been achieved for the first time and has allowed for phylogenetic relationships to be established using multiple gene loci. The increased use of multiple gene loci for phylogenetic analysis and molecular characterisation is recommended to allow for a more accurate view of the evolutionary relationships among the piroplasms to be established.

Study conducted within this thesis has highlighted the current discordance and general confusion in the taxonomic allocation among the canine piroplasms and within the order Piroplasmida at a species, genus and family level of classification (Chapters ten and eleven). The establishment of specific criteria for determining different levels of taxonomic allocation is overdue within this important group of protozoan parasites. A general consensus of criteria would minimize misguided and often premature descriptions of new piroplasm species. While traditional phenotypic characteristics should always be considered when classifying members of the Piroplasmida, genetic characterisation and phylogeny shows promise as a means of delineating taxa. By reorganising the order Piroplasmida into three families, the Theileriidae, Babesiidae and Piroplasmiidae and the establishment of new genera including the *Piroplasma* and *Achromaticus*, an attempt has been made to alleviate the taxonomic anomalies and paraphyly that currently exist.

12.3 Conclusion

Overall, this thesis has revealed the benefits of molecular-based techniques to monitor, manage and control emerging canine tick-borne disease, while also giving a greater insight into evolutionary relationships and taxonomic classification of these organisms. The

increased application of PCR in veterinary diagnosis will not only allow for increased diagnostic accuracy but has the potential to be implemented in the quarantine screening of imported animals. Combined with other detection methods, molecular technology will help to ensure the high levels of biosecurity of countries such as Australia and New Zealand.

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APPENDIX A

Response to NZMAF Document: Amendment to all Canine Import Health Standards: *Babesia gibsoni*

By Peter Irwin[1], Ryan Jefferies[1] and John Jardine[2]
1) School of Veterinary and Biomedical Sciences, Murdoch University, Murdoch, WA, 6150
2) VETPATH Laboratory Services P.O. Box 18, Belmont, WA, 6984

Our research of naturally infected and experimental cases of *Babesia gibsoni*, to be published later this year, addresses some of the aspects of *B. gibsoni* epidemiology and diagnosis that are critical to assessing import risk. However, due to limited funding and ethical considerations, exhaustive studies (e.g. experimental infection under 'natural' conditions [tick transmission or fighting] and the use of large numbers of dogs) have not been possible. Our responses to certain aspects of the NZMAF document are given below.

- PCR should always be performed concurrently with IFAT, and vice versa. Dogs with chronic *B. gibsoni* infection may be PCR negative in our experience, yet the vast majority of these will be seroreactive. The fact that PCR alone is not an adequate screen is noted in the document (2nd page), so we therefore cannot see any logic for the recommended 30 day test with PCR alone.

- We agree that PCR should replace blood examination.

- Persisting with an IFAT cut-off titre of 1:40 will continue to detect non-specific reactions. We will be publishing data to suggest that increasing the IFAT cut-off titre and/or co-testing at 1:40 and 1:160 (while concurrently testing with PCR) will remove these false positives and also reduce the chance of detecting cross reaction with *B. canis*.

- With the testing protocol as proposed, dogs with *B. canis* would be prohibited from travelling. In the section "Other Requirements" it is stated that "appropriate primers" must be used for *B. gibsoni*. We suggest:

  1) The use of a PCR capable of amplifying all members of the genera *Babesia* and *Theileria* with an additional speciation step (eg RFLP) to determine the species present. This will facilitate the detection of all canine piroplasm species including *B.*

gibsoni (Asian genotype), *Theileria annae*, un-named piroplasm species (Californian genotype) and *Babesia canis*.

2) The use of either nested or semi-nested PCR, allowing for increased sensitivity of the PCR procedure.

- As the document notes, short stay dogs <10 days that are never tested theoretically pose a risk for *B. gibsoni* entry into New Zealand. There is little doubt that biting is the main mode of transmission of *B. gibsoni* between dogs in certain countries (including Australia). We therefore recommend that either such short stay without testing is not allowed, or that these dogs are tested by both PCR and IFAT 20-30 days after travelling. In addition, these dogs should be restricted in their movements and contact with other dogs during this time.

- The time requirement for acaricide treatment seems excessive and appears to be based on data reflecting visual/microscopic detection of parasitaemia. In our experiments the dogs seroconverted between 1 and 3 weeks post-infection and remained positive for the duration of the experiment. Although these are experimental data, it appears that direct transmission may result in earlier detection of positive cases with IFAT and PCR. Under these circumstances consideration should be given to beginning the acaricide treatment at the date of the first PCR/IFAT blood test and continuing through until departure.

APPENDIX B

Australasian Centre for Companion Animal Research

Division of Health Sciences

School of Veterinary Biology & Biomedical Sciences

MURDOCH UNIVERSITY
PERTH, WESTERN AUSTRALIA

Dear Owner,

As part of our research into tick-transmitted diseases of dogs, we are currently investigating canine Babesiosis in Australia. This disease is caused by the blood parasite, *Babesia gibsoni* and can cause severe anaemia and sickness in dogs. Our research aims to gain an increased understanding of the distribution and prevalence of this disease and will help in better control and treatment.

By completing the following questionnaire you will be helping with this much needed research.

Babesia gibsoni in American Pit Bull Terriers in Victoria

Owner Questionnaire

OWNER CODE_____

DOG CODE/NAME_____

Breed (if different to American Pit Bull Terrier)_____

Age_____

Sex_____

Housing

- ☐ Individually penned
- ☐ Group penned
- ☐ Free run
- ☐ Other (please specify)_____

Contact with other dogs

Number of other dogs on property?_____

Does the dog ever mix with other dogs from different properties/owners?

☐ Yes ☐ No

Breeding and Travel history

Did you breed this dog? ☐ Or was it from another breeder? ☐

Has the dog ever travelled:

Interstate ☐ Specify state/s_____

Internationally ☐ Specify country_____

Health	Yes	No
Has the dog ever been bitten by another dog?	☐	☐
Has your dog ever had a blood transfusion?	☐	☐
Have you ever seen ticks on the dog?	☐	☐
Has the dog been treated for ticks?	☐	☐

If yes, which treatment?_____

Thankyou for your time and your help is greatly appreciated!

Ryan Jefferies, A/Prof Una Ryan and Dr Peter Irwin

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